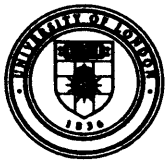

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Behavioural & Neurochemical Abnormalities in NK1 receptor Knockout (NK1R-/-) Mice: A Novel Rodent Model of Attention Deficit / Hyperactivity Disorder

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I hereby declare that the work described in this thesis has been initiated, performed and completed all by myself.

Abstract

NK1 receptor knockout (NK1R-/-) mice (which lack functional substance P-preferring NK1 receptors) have: (i) impaired regulation of noradrenaline (NA) release by α_2-adrenoceptors, and (ii) disrupted response to the rewarding effect of the psychostimulant, *d*-amphetamine (*d*-AMP). In the course of investigating whether this atypical response to *d*-AMP could be explained by their abnormal NA transmission, it was discovered that these mice display behavioural and neurochemical features that echo the core symptoms of Attention Deficit / Hyperactivity disorder (ADHD).

The project went on to test the validity of NK1R-/- mice as a model of ADHD, using *in vivo* microdialysis and the Light / Dark Exploration Box (LDEB). Changes in extracellular concentration ('efflux') of monoamines and the behavioural response of NK1R-/- mice were compared with the wild-type (NK1R+/+), following an acute administration of *d*-AMP or methylphenidate (MPH), which are both the first-line treatments for ADHD. Further, the possibility that a lack of functional NK1 receptors in NK1R-/- mice could be a causal factor for these abnormalities was tested, using an acute administration of an NK1R antagonist: RP 67580 or L 733060.

In the LDEB test, *d*-AMP and MPH increased locomotor activity of NK1R+/+ mice, but prevented the hyperactivity of NK1R-/- mice. In the microdialysis studies, basal dopamine (DA) efflux in the dorsal striatum was similar in both genotypes. After acute systemic injection of *d*-AMP, striatal DA efflux was increased in NK1R+/+, but not NK1R-/-, mice. This could help explain why psychostimulants do not increase arousal in ADHD. In the prefrontal cortex (PFC), basal DA efflux was > 50% lower in NK1R-/- mice, echoing the hypofrontality of ADHD patients. Systemic administration of *d*-AMP had no effect on cortical DA efflux in either genotype. Further, basal NA efflux in the PFC did not differ in the two genotypes, but declined in NK1R-/-, not NK1R+/+, mice when confined in the stressful light zone of the LDEB. This might echo the inattentiveness in ADHD. Finally, all these genotype differences were abolished by acute administration of an NK1R antagonist, RP 67580 or L 733060, which had no effect in NK1R-/- mice. This suggests that disruption of NK1 receptors, rather than compensatory adaptive change(s) in other neuronal systems, underlies the atypical responses of NK1R-/- mice.

Collectively, the striking abnormalities of NK1R-/- mice are consistent with impaired cortico-striatal catecholamine transmission in ADHD. These findings consolidate the validity of the mutants as a rodent model of this condition, and point to the use of NK1R agonists as a novel class of drug therapy.

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Abbreviations

aCSF	artificial cerebrospinal fluid
ANCOVA	analysis of covariance
ANOVA	analysis of variance
CNS	central nervous system
CPP	conditioned place preference
DA	dopamine
DAT	dopamine transporter
DβH	dopamine-β-hydroxylase
DRN	dorsal raphe nucleus
ECD	electrochemical detector
fmol	femtomole
fMRI	functional magnetic resonance imaging
FR2	frontal area 2
GABA	γ-aminobutyrate
h	hour
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine or serotonin
i.c.v.	intracerebroventricular
i.d.	inner diameter
i.p.	intraperitoneal
kD	kilodalton
Ki	inhibition constant
LC	locus coeruleus
LDEB	light / dark exploration box
M2	secondary motor cortex
mg	milligram
min	minute
µl	microlitre
µM	micromolar
mm	millimetre

mRNA	messenger ribonucleic acid
N	sample size
NA	noradrenaline
NAT	noradrenaline transporter
NAc	nucleus accumbens
NK1	neurokinin 1
nM	nanomolar
OCT	organic cation transporter
o.d.	outer diameter
6-OHDA	6-hydroxydopamine
P	probability
PCR	polymerase chain reaction
PGi	paragigantocellularis
PrH	prepositus hypoglossi
PFC	prefrontal cortex
s.c.	subcutaneous
SEM	standard error of means
SHR	spontaneous hypertensive rat
SN	substantia nigra
SPECT	single photon emission computed tomography
STN	subthalamic nucleus
TH	tyrosine hydroxylase
V	volt
VMAT	vesicular monoamine transporter *or* vesicular membrane associated transporter
VTA	ventral tegmental area

Publications arising from this thesis

Yan TC, Hunt SP & Stanford SC (2005). Comparison of regulation of K^+- and *d*-amphetamine-induced noradrenaline release in NK1R+/+ and NK1R-/- mice. *http://www.pa2online.org/abstract/search.jsp (pA_2 Online ISSN 1741-1157).*

Yan TC, Hunt SP & Stanford SC (2006). The effects of *d*-amphetamine and methylphenidate on behavioural responses of NK1R+/+ and NK1R-/- mice in the light/dark exploration box. *Journal of Psychopharmacology.* **20** (5): MA14.

Yan TC, Hunt SP & Stanford SC (2007). Investigation and validation of NK1R-/- mice as a novel rodent model of ADHD. *Journal of Psychopharmacology.* **21** (7): MD02.

Yan TC, Hunt SP & Stanford SC (2007). NK1R-/- mice: a novel rodent model of Attention Deficit Hyperactivity Disorder. *Program No. 386.1. Abstract Viewer/Itinerary Planner, Society for Neuroscience, San Diego, CA, 2007 (Online).*

Fisher AS, Stewart RJ, Yan T, Hunt SP & Stanford SC (2007). Disruption of noradrenergic transmission and the behavioural response to a novel environment in NK1R-/- mice. *European Journal of Neuroscience.* **25** (4): 1195–1204.

Yan TC, McQuillin A, Thapar A, Asherson P, Hunt SP, Stanford SC & Hugh G (2008). Phenotype deficits in NK1 (TACR1) receptor 'knockout' mice predict genetic association between the human TACR1 gene and attention deficit hyperactivity disorder. *In preparation.*

Chapter 1. General Introduction

Substance P is a neuropeptide that has been extensively studied since its characterization in 1970 (Chang & Leeman, 1970; see Werge, 2007). This peptide modulates many physiological and pathological processes by activating its preferred, G protein-coupled, NK1 receptors (NK1R). These receptors are predominantly located on the cell surface membrane (Yokota *et al*, 1989; Grady *et al*, 1996). However, internalization of these receptors evoked by substance P has also been observed both in transfected cells (Garland *et al*, 1994) and *in vivo* (Mantyh *et al*, 1995), a process contributing to rapid desensitization of cellular responses to the peptide.

In recent years, NK1 receptors have gained attention in the context of depression and anxiety. There is preclinical (Rupniak *et al*, 2001a; Santarelli *et al*, 2001) and clinical evidence (Kramer *et al*, 1998) that NK1R antagonists could be a novel class of antidepressant / anxiolytic therapies (see Section 1.3.3.1). Since all established antidepressants / anxiolytics exert their therapeutic effects by modulating monoamine transmission in the brain, this project was set out to investigate whether transmission of central noradrenaline (NA) and dopamine (DA) is abnormal in free-moving NK1R-/- mice (which lack functional, substance P-preferring, NK1 receptors), and whether such neurochemical abnormalities (if any) could account for the atypical behaviour of the mutants.

In the course of this study, we serendipitously discovered that many behavioural and catecholaminergic abnormalities of NK1R-/- mice echo core features of Attention Deficit Hyperactivity Disorder (ADHD), one of the most commonly developed mental disorders in children. Therefore, the results reported in this thesis offer evidence to support the use of NK1R-/- mice as a novel rodent model of this condition.

In this chapter, the background to this work is described. In particular, it depicts:

- The neuroanatomy of the prefrontal cortex (PFC) and the dorsal striatum, and their role in modulating motor behaviour
- Catecholamine transmission in the PFC and the dorsal striatum
- How catecholamine transmission can be modulated by NK1 receptors
- ADHD: subtype / treatments / neurobiology / animal models

1.1. The neuroanatomy of the prefrontal cortex and the dorsal striatum

1.1.1. The prefrontal cortex (PFC)

The frontal cortex can be divided into (i) the primary motor, (ii) the premotor and (iii) the prefrontal (located more rostrally than the former two regions) cortices. The mammalian PFC has been defined generally by various anatomical criteria or a combination of them:

A). In humans, the PFC is granular, whereas the (pre)motor cortices are agranular, as shown by cytoarchitectonic studies (Brodmann, 1909). However, later anatomical studies in primates revealed that several areas identified as granular by Brodmann (1909) do not contain a clear granular layer IV (e.g. Barbas & Pandya, 1989). Thus, cytoarchitectonic characteristics do not provide unequivocal criteria to delimit the PFC from other frontal cortical areas.

B). The PFC has specific reciprocal connectivity with the mediodorsal thalamic nucleus, as shown by studies using rabbits and cats (Rose & Woolsey, 1948), primates (Akert, 1964; Goldman-Rakic & Porrino, 1985), and rats (Groenewegen, 1988).

C). DA inputs from the ventral mesencephalon project exclusively to the PFC, based on various studies in rats (Thierry *et al*, 1973; Lindvall *et al*, 1978; Emson & Koob, 1978).

The PFC is not a homogenous structure. It can be subdivided into three topologically different regions (Sarter & Markowitsch, 1983; Divac & Mogenson, 1985; Sesack *et al*, 1989; for review, see Leonard, 1972; Groenewegen, 1988):

a). The medial PFC: a medially located cortical region, which constitutes the major portion of the medial wall of the hemisphere anterior and dorsal to the genu of the corpus callosum.

b). The orbital PFC: a ventrally located cortical region, which lies in part dorsal to the caudal end of the olfactory bulb in the dorsal bank of the rhinal sulcus.

c). The agranular insular cortex: a laterally located cortical region, which in rats is located in the anterior part of the rhinal sulcus.

The medial PFC, which is the part of the PFC particularly important for cognition (Amodio & Frith, 2006) and emotions (Lane *et al*, 1997; Gusnard *et al*, 2001), can be separated further into distinct areas, at least in rats (see Table 1.1):

Division based on cytoarchitonics (Krettek & Price, 1977; Van Eden & Uylings, 1985; see Heidbreder & Groenewegen, 2003)	**Division based on neuroanatomy** (Berendse *et al*, 1992; see Steketee, 2003)
i). The medial precentral area or area Fr2 ii). The anterior cingulate area iii). The prelimbic area iv). The infralimbic area	i). A dorsal component (consists of the Fr2, dorsal anterior cingulate areas, and the dorsal part of the prelimbic area): processes cognitive, i.e. the sensory aspects of the stimulus ii). A ventral component (consists of the ventral prelimbic, infralimbic and medial orbital areas): mainly innervated by the limbic system

Table 1.1 Subdivisions of the rat medial PFC

In this project, the M2 region of the mouse medial PFC, which appears to be anatomically equivalent to the Fr2 region in the rat dorsomedial PFC (Paxinos & Watson, 1998; Paxinos & Franklin, 2001), was studied. This brain area was chosen based on its anatomical arrangement and functions, which is discussed later in this Chapter.

Reciprocal projections exist between the PFC and many parts of the brain (see Fig 1.2). These brain areas include the thalamic nuclei and the ventral mesencephalon (as discussed above) as well as the brainstem (e.g. the locus coeruleus, see Section 1.2.1.1). The major type of neurone in the PFC is the pyramidal neurone, which comprises ~ 80% of the cortical neurones (see Fuster, 1989). They project from the PFC to the efferent targets, where glutamate is released as the major neurotransmitter (Fonnum *et al*, 1981; see Fonnum, 1984).

1.1.2. The dorsal striatum

Brain areas innervated by the PFC do not always send reciprocal projections back to the cortex. An example is the **dorsal striatum**. This basal ganglia region consists of the caudate nucleus and the putamen, and is separated from the nucleus accumbens (NAc; a part of the ventral striatum) based on anatomical and functional differences (see Section 1.2.1.2). The dorsal striatum receives direct projections from various parts of the PFC, including the Fr2 region in rats (Cheatwood *et al*, 2003; see Heidbreder & Groenewegen, 2003). However, this subcortical region does not project directly back to the PFC, but indirectly in parallel basal ganglia-thalamocortical circuits (see Fig 1.1), as shown by the tracing studies in primates (Alexander *et al*, 1986, 1990; Alexander & Crutcher, 1990) and rats (Groenewegen *et al*, 1990).

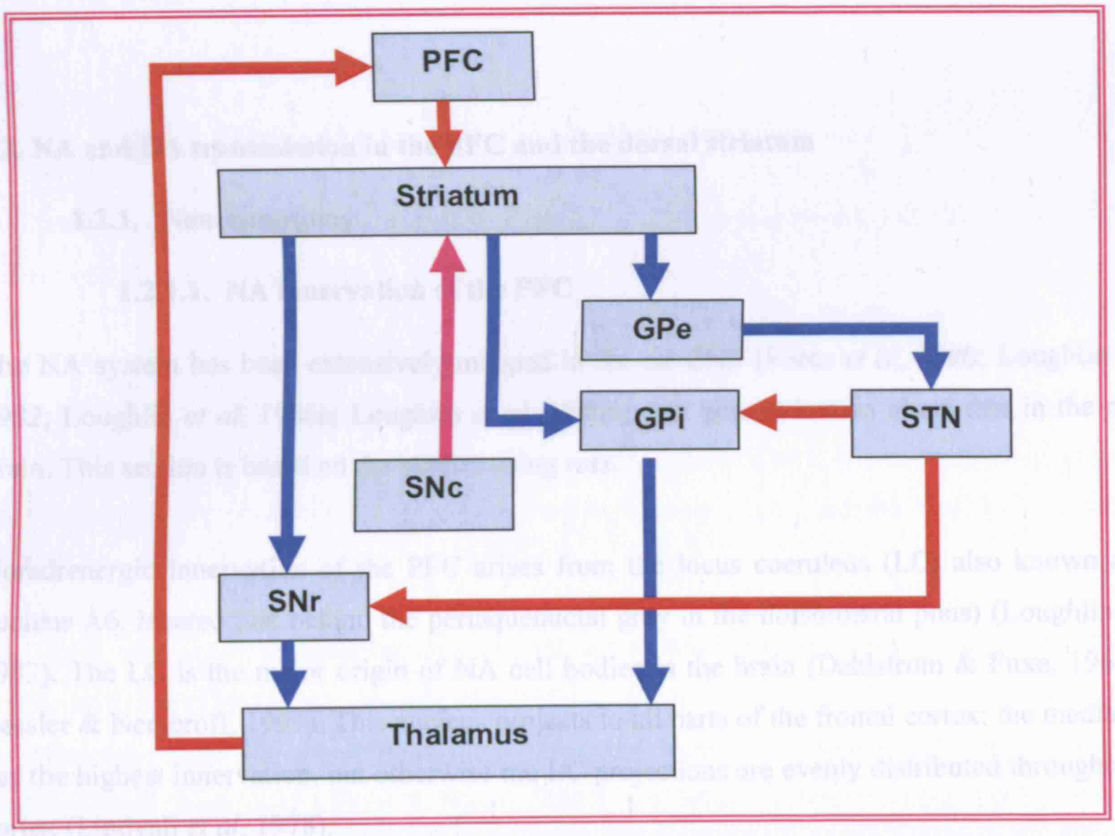


Fig 1.1 Diagram illustrating connectivity within basal ganglia-thalamocortical circuits. Red arrows: glutamatergic pathways; Blue arrows: GABA pathways; Pink arrows: the nigrostriatal DA pathway. GPe: globus pallidus (external segment); GPi: globus pallidus (internal segment); PFC: prefrontal cortex; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata; STN: subthalamic nucleus.

The dorsal striatum is innervated not only by cortical glutamatergic projections, which is the major input for this subcortical area (see Wilson, 1987), but also by nigrostriatal DA projections from the substantia nigra (SN; the A9 DA cell group).

The major cell type in the dorsal striatum is the striatal spiny neurone, which accounts for ~ 95% of total cells in this brain area (Kemp & Powell, 1971). These striatal neurones project to various parts of the basal ganglia, including the SN, where GABA is released as the major neurotransmitter (Kita & Kitai, 1988). Yet, there is also evidence from tracing studies that a population of striatal spiny neurones that projects to the SN expresses substance P, and releases this neuropeptide together with GABA in the terminals (Lee *et al*, 1986; Gerfen & Yound, 1988; Lee *et al*, 1997).

1.2. NA and DA transmission in the PFC and the dorsal striatum

1.2.1. Neuroanatomy

1.2.1.1. NA innervation of the PFC

The NA system has been extensively mapped in the rat CNS (Foote *et al*, 1980; Loughlin *et al*, 1982; Loughlin *et al*, 1986a; Loughlin *et al*, 1986b), but little is known about that in the mouse brain. This section is based on the studies using *rats*.

Noradrenergic innervation of the PFC arises from the locus coeruleus (LC; also known as the nucleus A6, located just behind the periaqueductal gray in the dorsorostral pons) (Loughlin *et al*, 1982). The LC is the major origin of NA cell bodies in the brain (Dahlstrom & Fuxe, 1964; see Ressler & Nemeroff, 1999). This nucleus projects to all parts of the frontal cortex: the medial PFC has the highest innervation, but otherwise the LC projections are evenly distributed throughout the cortex (Lindvall *et al*, 1978).

The LC also receives innervation from the medial PFC, as demonstrated by the anterograde labeling studies in monkeys (Arnsten & Goldman-Rakic, 1984) and rats (Sesack *et al*, 1989).

However, inconsistent evidence has emerged regarding the influence of the cortical region on activity of the LC. The electrophysiological study in rats by Sara and Herve-Minvielle (1995) revealed a tonic inhibition of the LC neuronal activity by neurones in the dorsomedial Fr2 region of the PFC. In contrast, Aston-Jones and colleagues suggested that such influence would to be excitatory, as electrical or chemical stimulation of the PFC with L-glutamate activates LC neurones in anaesthetized rats (Jodo *et al*, 1998).

In contrast to the PFC, there is so far no anatomical evidence that the dorsal striatum receives NA projections (Lindvall & Bjorklund, 1983; personal communication with Prof. Thomas Hökfelt, Karolinska Institute, Stockholm, Sweden), although immunoreactivity for the NA synthetic enzyme, dopamine-β-hydroxylase (DBH), has been found in the shell sub-region of the ventral striatum (Berridge *et al*, 1997).

1.2.1.2. DA innervation of the PFC and the dorsal striatum

The central DA system is topographically divided into the mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular pathways.

The **mesocortical** pathway consists of DA projections to the PFC from the ventral tegmental area (VTA; the A10 nucleus, located in the midbrain). In contrast to the diverse cortical projections of the LC NA neurones, the mesocortical DA neurones innervate only relatively limited regions in the cortex, such as the medial PFC (as discussed in Section 1.1.1; Lindvall *et al*, 1978; Descarries *et al*, 1987; see Marsden, 2006).

The VTA also sends **mesolimbic** DA projections to brain regions closely associated with the limbic structures: most prominently the NAc (Dahlstrom & Fuxe 1964; Ungerstedt, 1971). Anatomically, there is not a clear separation of mesolimbic and mesocortical systems. In fact, in some authors' view (e.g. Moore & Bloom, 1978; see White, 1996; Marsden, 2006), these two circuits are considered as a whole system, known as the *mesocorticolimbic* DA system.

There is a general consensus that the mesocorticolimbic system is separated from the **nigrostriatal** DA system, which projects from the lateral SN to the dorsal striatum (see Fallon, 1988) (see

Fig 1.2). However, there is autoradiographical evidence showing cross-over of the two systems, such that the PFC innervated by the VTA projects to the same part of the dorsal striatum as the VTA, and the PFC innervated by the SN projects to the same part of the dorsal striatum as the SN (see Beckstead, 1979).

The **tuberoinfundibular** pathway runs from the arcuate and periventricular nuclei of the hypothalamus to the intermediate lobe of the pituitary and the median eminence (see Marsden, 2006). This system inhibits release of growth hormone, which could induce important side effects when ADHD children are treated with medications that enhance DA transmission in this pathway.

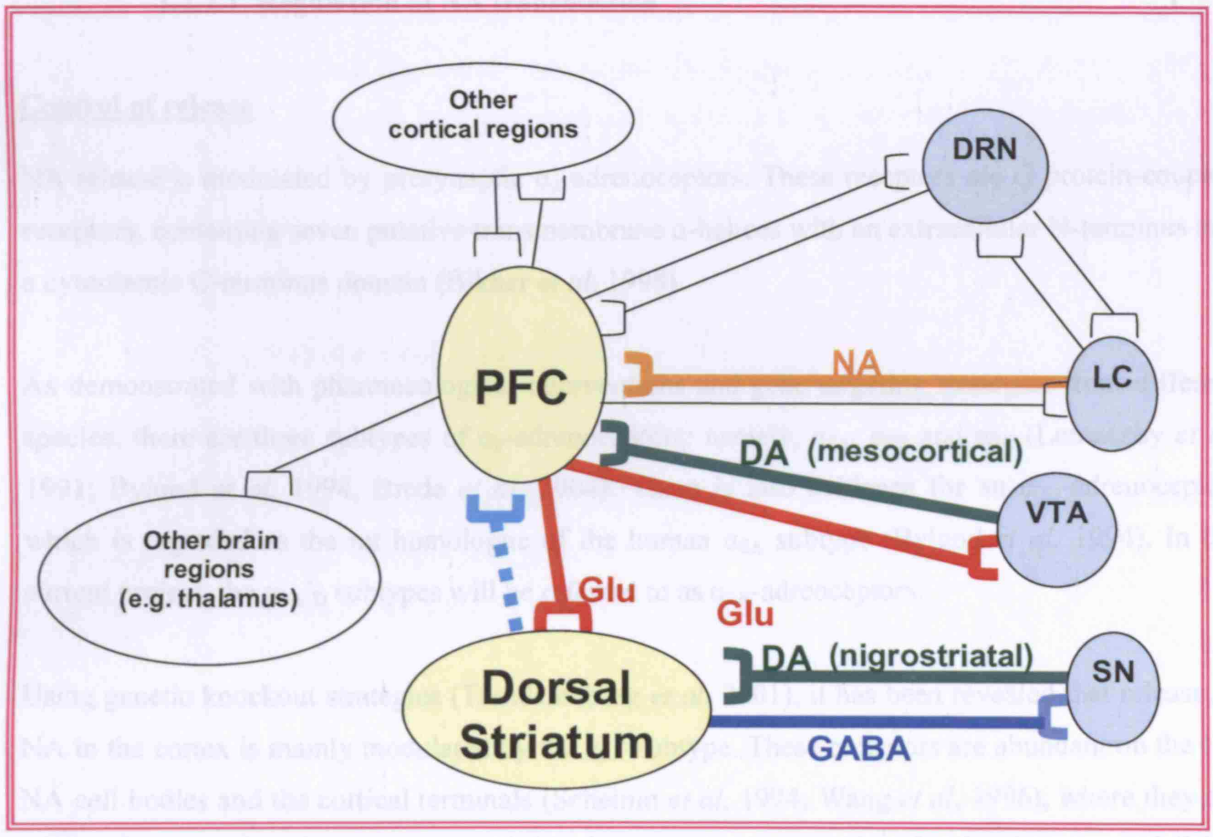


Fig 1.2 Neurochemical links between the PFC, the dorsal striatum, and their key input/output nuclei. Links relevant to this project are highlighted. Solid lines: directed projections. Dotted line: indirect projections from the dorsal striatum to the PFC through various basal ganglia nuclei. PFC: prefrontal cortex; LC: locus coeruleus, SN: substantia nigra, VTA: ventral tegmental area; DRN: dorsal raphe nucleus; Glu: glutamate.

1.2.2. Regulation of NA and DA transmission in the PFC and the dorsal striatum

In the current study, *in vivo* microdialysis was used to monitor changes in extracellular concentrations ('***efflux***') of catecholamines. Efflux is the amount of extracellular transmitter that spills over from the synapse into the microdialysis probe, hence serving as an index of neurotransmission (see Chapter 2 for details). Efflux depends on the balance between ***release*** and ***reuptake***. In this section, how these two factors regulate NA and DA efflux in the PFC and the dorsal striatum is described.

1.2.2.1. Regulation of NA transmission

Control of release

NA release is modulated by presynaptic α_2-adrenoceptors. These receptors are G protein-coupled receptors, containing seven putative transmembrane α-helices with an extracellular N-terminus and a cytoplamic C-terminus domain (Bikker *et al*, 1998).

As demonstrated with pharmacological interventions and gene targeting strategies from different species, there are three subtypes of α_2-adrenoceptors: namely, α_{2A}, α_{2B} and α_{2C} (Lomasney *et al*, 1991; Bylund *et al*, 1994; Brede *et al*, 2004). There is also evidence for an α_{2D}-adrenoceptor, which is regarded as the rat homologue of the human α_{2A} subtype (Bylund *et al*, 1994). In the current project, the $\alpha_{2A/D}$ subtypes will be referred to as α_{2A}-adreoceptors.

Using genetic knockout strategies (Trendelenburg *et al*, 2001), it has been revealed that release of NA in the cortex is mainly modulated by the α_{2A}-subtype. These receptors are abundant on the LC NA cell bodies and the cortical terminals (Scheinin *et al*, 1994; Wang *et al*, 1996), where they act as autoreceptors that decrease extracellular concentrations of NA by blunting its neuronal firing and reducing its release. In contrast, cortical NA transmission is less (if at all) regulated by the α_{2B} or α_{2C} subtypes, which reside in the plasma membrane in the thalamus (α_{2B}) and intracellularly in mainly the basal ganglia (α_{2C}), respectively (Scheinin *et al*, 1994).

Control of reuptake

NA reuptake is carried out predominantly by the NA transporter (NAT), although uptake *via* other transporters (e.g. the organic cation transporter; OCT: Russ *et al*, 1996; Wu *et al*, 1998; Breidert *et al*, 1998) is also possible. The NAT is a Na^+ / Cl^--dependent 12 transmembrane-spanning protein residing on presynaptic NA nerve terminals (see Amara & Kuhar, 1993; Blakely *et al*, 1994; Amara *et al*, 1998).

As shown by autoradiography studies in rats, while expression of the NAT is low in the striatum, it is high in the frontal cortex (Sanders *et al*, 2005). Therefore, in the cortical region, termination of NA transmission mainly occurs *via* the reuptake mediated by the NAT (see Blakely *et al*, 1994; Géranton *et al*, 2003a). This role of the NAT is well demonstrated by knockout technologies *in vivo*. By comparing mice that lack functional NAT gene (NAT-/-) with their wild-type counterparts, Xu *et al* (2000) reported profound alterations in presynaptic homeostasis in the mutants: they show less total tissue NA content and prolonged clearance of NA in the PFC, hence leading to a 2-fold increase in cortical NA efflux.

1.2.2.2. Regulation of DA transmission

Control of release

As with NA, release of DA is exquisitely regulated by its autoreceptors. These autoreceptors, consisting of the D2, D3 and D4 subtypes, are G-protein-coupled receptors that belong to the D2-family of DA receptors (see Schmitz *et al*, 2003).

Immunolabelling and mRNA expression of these autoreceptors are similar in the rat (Sesack *et al*, 1994) and the human brain (Meador-Woodruff *et al*, 1996). D2 and D3 receptors are most abundant in the limbic system. In particular, the **D2** subtypes are highly expressed by somata and dendrites of DA neurones in the SN and the VTA, as well as by their terminals in the dorsal striatum and the NAc. While the **D3** subtype immunoreactivity is also expressed by the midbrain DA cell bodies and in the NAc, it is not found in the dorsal striatum (Diaz *et al*, 2000). The distribution of **D4** subtypes is different. These receptors, unlike other D2-family members, are highly expressed in the PFC. Inside the cortical region, D4 receptors are present on both excitatory

glutamatergic pyramidal neurones (Rubinstein *et al*, 2001) and the inhibitory GABAergic interneurones (Mrzljak *et al*, 1996), where they act as an inhibitory modulator of activity of these transmitters.

Regulation of DA transmission is predominantly carried out by the D2 subtype, with a relatively minor contribution from the D3 subtype, and none from D4 receptors. This is supported by animal studies which showed that activation of D2 receptors inhibits **release** of DA from terminals (*in vitro* slice studies: Cubeddu & Hoffmann, 1982; Mayer *et al*, 1988; Cragg & Greenfield, 1997; *in vivo* voltammetric study: Stamford *et al*, 1988; May & Wightman, 1989) and from somatodendrites (Cragg & Greenfield, 1997). Further, DA **neuronal firing rate** is decreased by D2 receptor activation (Bunney *et al*, 1973; Silva & Bunney 1988; Rayport *et al*, 1992; Mercuri *et al*, 1997), but is enhanced by D2 receptor antagonism *in vivo* (Bunney & Grace, 1978). In comparison, studies on transfected DA cell lines revealed that D4 receptors do not regulate DA release or synthesis (Tang *et al*, 1994; O'Hara *et al*, 1996), hence these receptors are most likely postsynaptic.

Control of reuptake

The DA transporter (DAT), like the NAT, is a member of the family of biogenic amine transporters with twelve transmembrane domains (see Blakely & Bauman, 2000; Torres *et al*, 2003). These transporters are most densely localized on the DA terminals in the dorsal striatum and the NAc (Marshall *et al*, 1990). In these brain regions, DA reuptake is the most important mechanism of inactivation of DA signaling (Jones *et al*, 1998a; Benoit-Marand *et al*, 2000). Moreover, the DAT expression is also found on cell bodies and dendrites in the SN and VTA (Nirenberg *et al*, 1996).

The DAT plays an important role in regulating DA transmission. Microdialysis studies showed that drugs which block the DAT (e.g. cocaine and methylphenidate) augment DA efflux in rats (e.g. Kuczenski *et al*, 1991; Kuczenski & Segal, 1997). Moreover, in DAT knock-out (DAT-/-) mice, a model of ADHD (see Section 1.4.4.2), altered regulation of DA transmission is observed: including a reduction (-95%) of striatal DA content and a 5-fold increase in DA efflux in the dorsal striatum (Giros *et al*, 1996; see Gainetdinov & Caron, 2003).

1.2.2.3. Crosstalk between central NA and DA systems

Interactions between central NA and DA systems occur. This is particularly the case in the medial PFC, where the two catecholaminergic projections converge (Fadda *et al*, 1984). Using microdialysis, concomitant changes in cortical NA and DA efflux have been observed in various conditions, e.g. during **stress** (Kawahara *et al*, 1999; Feenstra, 2000), or after administration of **psychostimulants** (Darracq *et al*, 1998) or **α_2-adrenoceptor agonists / antagonists** (Gresch *et al*, 1995; Devoto *et al*, 2001; Kawahara *et al*, 2001).

The concomitant changes in extracellular NA and DA in the medial PFC could be explained by the '**heterotransport hypothesis**', which suggests that reuptake of DA in the NA-rich brain area is mediated by the NAT. This theory is based on the fact that the NAT has a similar affinity for both catecholamines (*Km*=~1 μM; Giros *et al*, 1994), and is supported by the following evidence:

- There is microdialysis evidence in rodents that NAT inhibitors increase DA efflux in the PFC (Carboni *et al*, 1990; Yamamoto & Novotney, 1998).
- While the DAT inhibitor, GBR 12909, has no effect on DA uptake in the medial PFC *in vitro* (Morón *et al*, 2002) or *in vivo* (Mazei *et al*, 2002), inhibition of the NAT by desipramine caused a concomitant increase in both NA and DA efflux (Gresh *et al*, 1995).
- In NAT knockout mice, synaptosomal DA uptake is greatly reduced (Morón *et al*, 2002).

Another theory, termed the '**co-transmission hypothesis**' might also be applied. This theory suggests that DA in the medial PFC acts not only as NA precursor, but also as a co-transmitter which is released with NA from noradrenergic terminals. This hypothesis is based on various microdialysis findings from Devoto and colleagues, for example:

- DA efflux in the occipital and parietal cortices (which have scarce DA projections) is only slightly lower than that in the medial PFC (which has dense DA afferents). This suggests that DA in the cortex may be released from sites other than DA terminals (Devoto *et al*, 2001).

- Clozapine, which inhibits α_2-adrenoceptors, increases both NA and DA efflux in the medial PFC and the occipital cortex. This increase does not differ in the two brain areas, although they have different densities of DA terminals. Moreover, the effects of clozapine are reversed by the α_2-agonist clonidine, but not by the D2-agonist quinpirole. This therefore indicates that DA could be co-released with NA in the cortex (Devoto *et al*, 2003).

There could be other interpretations for the coupling between the two catecholamines in the PFC. For example, the LC NA neurones could activate mesocortical DA neurones in the VTA, and consequently lead to DA release from the mesocortical terminals in the PFC (Herve *et al*, 1982; Linner *et al*, 2001). Nevertheless, this might not be the case, as there is evidence that electrical stimulation of the LC NA neurones produces a long-lasting *inhibition* of the electrical activity of midbrain DA neurones (Grenhoff *et al*, 1993).

1.2.3. Modulation of the psychostimulant-induced motor activity by catecholamines in the PFC and the dorsal striatum

The turning point of this project was the serendipitous discovery of an abnormal motor response of NK1R-/- mice to the psychostimulant, *d*-amphetamine (*d*-AMP) (see Chapter 5). Therefore, although the PFC and the dorsal striatum are also known for their prominent roles in, for instance, cognitive functions, the current chapter describes only their functions in motor activity.

1.2.3.1. Modulation by DA in the dorsal striatum

There used to be a general consensus that the psychostimulant-induced locomotor activation was associated mostly with the drug-evoked DA release in the NAc, while DA release in the dorsal striatum was linked with stereotypy elicited by high doses of psychostimulants. This concept is based on the landmark studies from Iverson and colleagues, using rats whose DA stores in the NAc or the dorsal striatum are lesioned by bilateral injections of the neurotoxin, 6-OHDA, into these subcortical areas (Kelly *et al*, 1975; Kelly & Iversen, 1976). Subsequent microdialysis studies in freely-moving rats lent further support to this theory (e.g. Sharp *et al*, 1986).

Consistent findings have also been reported by the microdiaysis studies from Puglisi-Allegra and co-workers in freely-moving mice (the C57BL/6 strain) (Zocchi *et al*, 1998, Ventura *et al*, 2004). This group, to our best knowledge, is the only group that reported a *d*-AMP-induced increase in DA efflux in the *mouse* NAc. In fact, interpretation of such microdialysis data could be difficult, given that (i). the mouse NAc is tiny (dorsoventral diameter is < 1 mm: Paxinos & Franklin, 2001), and insertion of the microdialysis probe could induce severe damage of this structure; (ii) the mouse NAc is divided into the shell and the core, which are anatomically connected to distinct brain regions and are associated with different functions (Heimer *et al*, 1991; Deutch & Cameron, 1992; Pontieri *et al*, 1995), hence it is difficult to ascertain which part of the NAc is being dialyzed.

In fact, it is now believed that the neurochemical mechanisms underlying the ***motor-activating*** and ***stereotypic*** effects of psychostimulants are not clearly separated. This is because DA transmission in the dorsal striatum also plays a crucial role in motor functions, as demonstrated by the deficit in motor performance in mice treated with the DA neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (e.g. Quinn *et al*, 2007). Moreover, several studies revealed that striatal DA transmission contributes to the motor stimulating effect of psychostimulants. Ungerstedt (1971) found that, after unilateral lesions of the dorsal striatum with 6-OHDA in rats, systemic administration of *d*-AMP increases release of DA in the unlesioned side and ipsilateral rotations towards the lesioned side. In line with this, the Iversen group reported that bilateral 6-OHDA microinjections into the SN, where the nigrostriatal DA neurones are located, abolish both the locomotor *and* stereotypy responses to *d*-AMP in adult rats (Creese & Iversen, 1975). More recently, Rebec and colleagues revealed that both intrastriatal infusion (Wang *et al*, 1993) and systemic injection (s.c.) (Haracz *et al*, 1993) of *d*-AMP elicit motor activity in rats, and the systemic administration of the drug also increases single-unit responses in the dorsal striatum. Further, Antoniou *et al* (1998) showed that a 6-OHDA lesion of the dorsal striatum abolishes the *d*-AMP-induced locomotion in rats. Therefore, all these findings point to the important role of dorsal striatal DA transmission in mediating the psychostimulant-induced motor activation.

In this project, one of the important aims is to find out whether the locomotor response to *d*-AMP differs in NK1R+/+ and NK1R-/- mice and if so, whether this could be explained by a genotype

difference in the subcortical DA response to the drug. Hence, microdialysis has been carried out in the mouse dorsal striatum.

1.2.3.2. Modulation by DA and NA in the PFC

Mounting evidence has shown that the modulation of the psychostimulant-induced locomotion by subcortical DA transmission is under the influence of a complex neural network, involving other brain regions and neurotransmitters (Piazza & Le Moal, 1996; Darracq *et al*, 1998). The predominant innervation of the dorsal striatum is the glutamatergic projection from the PFC (Spencer, 1976; Carter, 1982, Barbieto *et al*, 1990). Hence, it is not surprising that the motor response to psychostimulants also depends on this cortical region. This is supported by early studies, which showed that primates with PFC lesions are hyperactive (Kennard *et al*, 1941; French, 1959; Gross, 1963).

The involvement of the PFC in motor functions is believed to be mediated by its modulation of DA transmission in the subcortical areas. The cortical glutamate neurones send efferent projections to subcortical areas, but this action could be *inhibited* by DA released from mesocortical dopaminergic terminals, as suggested by various lesion studies (Carter & Pycock, 1978, 1980; Taber *et al*, 1995). This process can either be a direct or an indirect one (see Fig 1.3). The *direct* inhibition of PFC glutamate by mesocortical DA occurs as DA projections that originate in the VTA, synapse directly on cortical glutamate neurones (Penit-Soria *et al*, 1987), and inhibit their activity possibly *via* DA D4 receptors (as shown by the study using D4 receptor-deficient mice: Rubinstein *et al*, 2001). DA can also inhibit glutamate *indirectly* by synapsing on GABAergic interneurones in this brain region (Penit-Soria *et al*, 1987; Sesack *et al*, 1995; Grobin & Deutch, 1998). As cortical glutamate neurones innervate subcortical structures, such as the VTA, the dorsal striatum and the NAc (Christie *et al*, 1985; Sesack & Pickel, 1992), DA released from the mesocortical terminals in the PFC may remove the subsequent glutamate excitation of DA neurones in the subcortical areas.

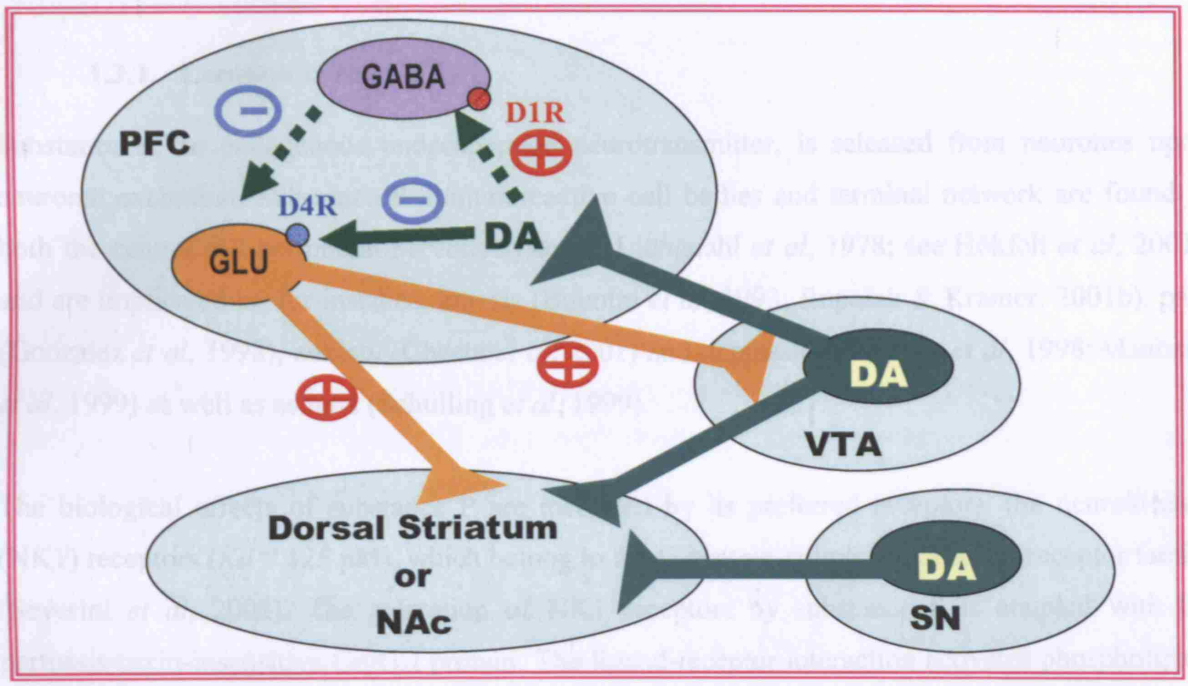


Fig 1.3 The inhibitory modulation of subcortical DA transmission by DA in the PFC. NAc: nucleus accumbens; PFC: prefrontal cortex; SN: substantia nigra; VTA: ventral tegmental area; GLU: glutamate. Solid arrow: direct inhibition of glutamate activity by PFC DA *via* the inhibitory D4 receptors. Dotted arrow: indirect inhibition of glutamate activity by PFC DA (D1 receptors are located on GABA interneurones, and their activation leads to increased release of GABA, hence subsequent inhibition of glutamate neuronal activity).

The PFC is heavily modulated not only by DA, but also by NA transmission (see Arnsten, 1997). In the PFC, the two catecholamines have close interactions (see Section 1.2.2.3). Hence, NA released in the cortex could also modulate the role of this brain area in motor performance. Supporting evidence comes from the recent microdialysis studies by Tassin and colleagues, who revealed that activation of α_1-adrenoceptors in the PFC is required for the expression of *d*-AMP-induced locomotor activation (Darracq *et al*, 1998; Auclair *et al*, 2002; see Section 6.5.3 for more details). NA efflux in the M2 PFC region (which has reciprocal connections with the LC, in rats at least) is greater in anaesthetized NK1R-/- mice (Herpfer *et al*, 2005; Fisher *et al*, 2007). Further, this brain region sends direct projections to the dorsal striatum and modulates DA transmission in this subcortical area (as discussed above). Therefore, whether the abnormal motor response of NK1R-/- mice to psychostimulants (if any) could also be explained by their atypical DA as well as NA transmission in the PFC M2 region was also tested in this project.

1.3. Substance P and NK1 receptors in the brain

1.3.1. Location in the CNS

Substance P, an endogenous undecapeptide neurotransmitter, is released from neurones upon neuronal excitation. Substance P-immunoreactive cell bodies and terminal network are found in both the central and peripheral nervous systems (Ljungdahl *et al*, 1978; see Hökfelt *et al*, 2001), and are implicated in, for instance, emesis (Bountra *et al*, 1993; Rupniak & Kramer, 2001b), pain (Gonzalez *et al*, 1998), anxiety (Cheeta *et al*, 2001) and depression (Kramer *et al*, 1998; Maubach *et al*, 1999) as well as asthma (Schuiling *et al*, 1999).

The biological effects of substance P are mediated by its preferred receptors, the neurokinin-1 (NK1) receptors (*Kd* = 125 pM), which belong to the G-protein-coupled tachykinin receptor family (Severini *et al*, 2002). The activation of NK1 receptors by substance P is coupled with the pertussis-toxin-insensitive Gq/G_{11} protein. The ligand-receptor interaction activates phospholipase C to breakdown phosphatidyl inositol bisphosphate into inositol trisphosphate (IP3) and diacylglycerol (DAG) (Grandordy *et al*, 1988). IP3 acts on Ryanodine receptors in the sarcoplasmic reticulum to cause release of Ca^{2+} from intracellular stores, while DAG acts *via* protein kinase C to open the membrane-bound L-type Ca^{2+} channels (Gallacher *et al*, 1990). The resultant increase in intracellular Ca^{2+} concentration therefore induces the tissue response to substance P. However, in a recent whole cell binding study, Holst *et al* (2001) showed that NK1 receptors not only form complexes with Gq proteins, but also with Gs protein, which leads to activation of adenylate cyclase and the subsequent increase in formation of cAMP. These authors also revealed that the complexes of NK1 receptor with either Gq or Gs protein do not readily interchange, suggesting these tachykinin receptors exist in different membrane microdomains.

As with substance P, NK1 receptors are observed in both the brain and periphery in autoradiographic studies (Mantyh *et al*, 1989). However, discrepancies of distribution of substance P and NK1 receptors occur in several brain regions (Ljungdahl *et al*, 1978; Mantyh et al, 1984a, b; Shults *et al*, 1984; see Otsuka & Yoshioka, 1993; see Table 1.2 and Fig 1.4):

Brain regions	Substance P	NK1 receptors
PFC	-	++
Dorsal striatum	+++	++++
SN	++++	-
VTA	++	-
LC	+++	+++
DRN	+++	++

Table 1.2 Distribution of substance P and NK1 receptors in the rat CNS, revealed by autoradiographic and immunohistochemical studies.

As detailed in Table 1.2, in the SN and the VTA, where the nigrostriatal and mesocortical DA cell bodies are located, high levels of mRNA expression for substance P are demonstrated, but these brain regions show very little (if any) expression of NK1 receptors. However, in the dorsal striatum, the terminal field of nigrostriatal DA neurones, highest level of NK1 receptor expression is observed. In this subcortical area, NK1 receptors are found on acetylcholine-producing interneurones, as is evident from *in situ* hybridization histochemistry and fluorescent retrograde tracing studies in rats (e.g. Gerfen & Young, 1988). Moreover, low to moderate expression of NK1 receptors is also seen in the PFC, where mesocortical DA projections terminate. In this cortical region, NK1-like immunoreactivity is mainly observed on non-pyramidal

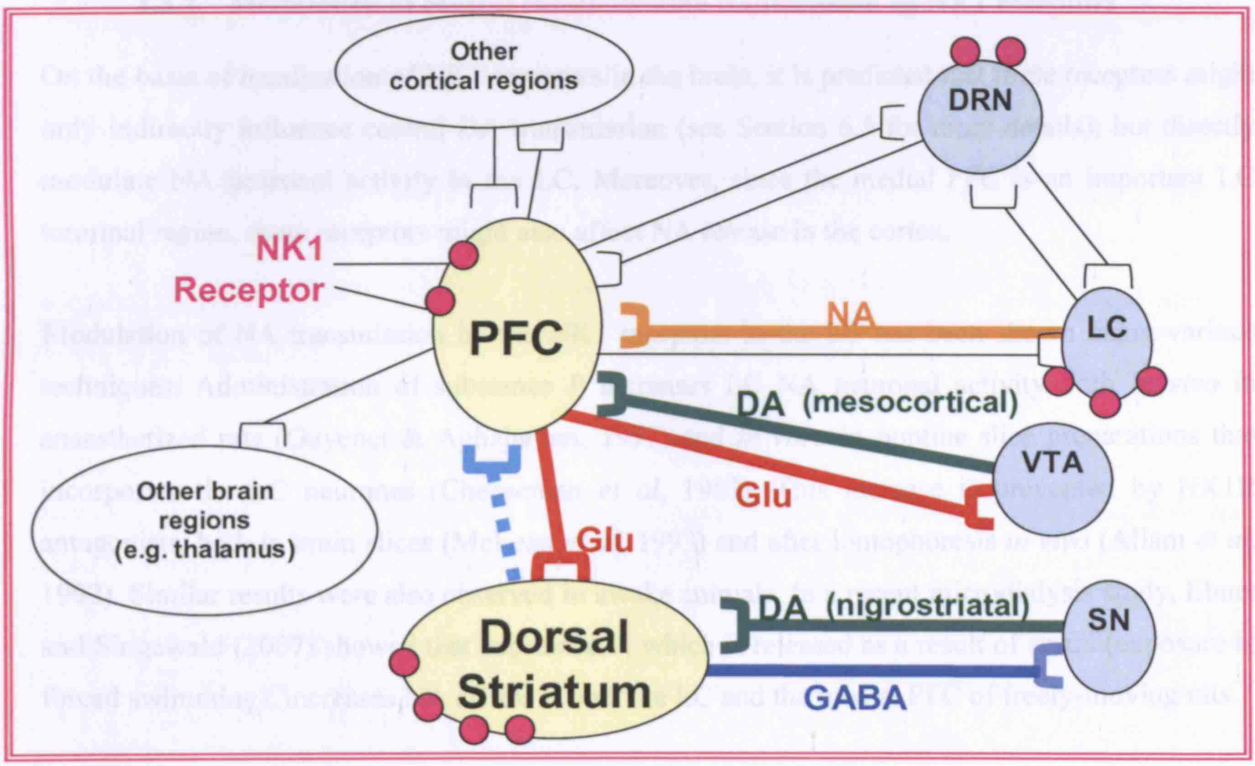


Fig 1.4 Localization of NK1 receptors in the rat CNS.

cells and in the neuropil of the PFC (Tooney *et al*, 2000). Therefore, in both the nigrostriatal and mesocortical DA systems, NK1 receptors are located *postsynaptically* to DA terminals.

Moreover, in the LC (the primary source of noradrenergic fibers in the brain), NA neurones are targeted by SP-containing fibers, which form axo-dendritic contacts with tyrosine hydroxylase-positive cells (Ljungdahl *et al*, 1978; Pickel *et al*, 1979). The LC also displays a dense expression of NK1 receptors on NA neurones, according to immunohistochemical (Hahn & Bannon 1999; Ma & Bleasdale, 2002) and immunofluorescence studies in rats (Chen *et al*, 2000).

Further, the DRN, where 5-HT cell bodies are located, also shows a good correlation between expression of substance P-immunoreactive cell bodies / terminals and that of NK1 receptors (see Otsuka & Yoshioka, 1993). There is immunohistochemical evidence that, in the DRN, NK1 receptors are mostly cytoplasmic in 5-HT neurones (Lacoste *et al*, 2006), although it is also colocalized with GABA interneurones in this brain region (Ma & Bleasdale, 2002).

1.3.2. Modulation of central catecholamine transmission by NK1 receptors

On the basis of localization of NK1 receptors in the brain, it is predicted that these receptors might only indirectly influence central DA transmission (see Section 6.5 for more details), but directly modulate NA neuronal activity in the LC. Moreover, since the medial PFC is an important LC terminal region, these receptors might also affect NA release in the cortex.

Modulation of NA transmission by the NK1 receptors in the LC has been shown using various techniques. Administration of substance P increases LC NA neuronal activity both *in vivo* in anaesthetized rats (Guyenet & Aghajanian, 1977) and *in vitro* in pontine slice preparations that incorporate the LC neurones (Cheeseman *et al*, 1983). This increase is prevented by NK1R antagonists, both in brain slices (McLean *et al*, 1993) and after iontophoresis *in vivo* (Allam *et al*, 1992). Similar results were also observed in awake animals. In a recent microdialysis study, Ebner and Singewald (2007) showed that substance P, which is released as a result of stress (exposure to forced swimming), increases NA efflux in both the LC and the medial PFC of freely-moving rats.

1.3.3. NK1 receptor knockout mice

NK1R-/- mice were originally generated by De Felipe *et al* (1998). Receptor autoradiography with [^{125}I]Bolton-Hunter substance P indicates that binding sites of the NK1 receptor preferred ligand, substance P, are absent in the brain of these mutants. Thus, there are no NK1 receptors available to mediate actions of the tachykinin in these animals, although their receptor mRNA is still present. The lack of functional NK1 receptors has been confirmed by immunohistochemistry using a specific antibody raised against the carboxy terminus of the NK1 receptor (De Felipe *et al*, 1998).

These mice can be considered as a model of whole-life treatment with selective NK1R antagonists. Hence, these mutants provide a valuable research tool in the studies investigating the potential use of NK1R antagonists in the treatment of psychiatric (e.g. depression and anxiety) and neurological disorders (e.g. pain and emesis) (see Rupniak & Kramer, 2001b).

1.3.3.1. Antidepressant / anxiolytic-like phenotype

In recent years, NK1R-/- mice have been used extensively in the investigations of depression / anxiety. These mutants show marked reduction in aggressive behaviour in the *resident intruder test* as well as a decrease in *maternal separation-induced neonatal vocalization*, compared with the wild-type. Moreover, these behaviours of NK1R-/- mice do not differ from those of NK1R+/+ mice that had been given antidepressants (e.g. fluoxetine) (De Felipe *et al*, 1998; Rupniak *et al*, 2000, 2001a; Santarelli *et al*, 2001). Nevertheless, the use of NK1 antagonists as novel antidepressants / anxiolytics has not translated into the clinic.

1.3.3.2. Abnormalities of NK1R-/- mice that lead to the current study

Previous studies from this lab have revealed the following behavioural and neurochemical abnormalities in NK1R-/- mice:

Hyperactivity: NK1R-/- mice showed increased locomotor activity in the light / dark exploration box (LDEB; see Chapter 2 for details of this paradigm) and in the activity meter, compared with their wild-type counterparts (Herpfer *et al*, 2005; Fisher *et al*, 2007).

Impaired NA transmission by α_2-adrenoceptors: previous microdialysis studies in ***anaesthetized*** animals revealed that NA efflux in the M2 PFC region was 2 ~ 4 fold greater in NK1R-/- than in NK1R+/+ mice (Stewart *et al*, 2004; Herpfer *et al*, 2005; Fisher *et al*, 2007). Since microdialysis

monitors changes in extracellular concentrations, a higher NA efflux could reflect a reduction of reuptake, an increase in release, or both. These possibilities have been tested as follows:

Herpfer et al (2005) showed that:

- Systemic administration of the NAT blocker, desipramine, increases NA efflux to a similar extent in both genotypes.
  ➔ Indicating that uptake by the NAT is not impaired in NK1R-/- mice.
- Local infusion of the α_2-adrenoceptor antagonist, RX 821002, into the PFC (by retrodialysis) increases NA efflux to a similar extent in both genotypes.
  ➔ Suggesting that the α_2-autoceptors *on the PFC terminals* function normally in NK1R-/-.
- Systemic pretreatment with desipramine augmented the RX 821002-induced increase in NA efflux in NK1R+/+, but not NK1R-/- mice.
  ➔ Leading to the question whether functions of the somatodendritic α_2-autoceptors in the LC are disrupted in NK1R-/- mice. This was confirmed by Fisher *et al* (2007).

Fisher et al (2007) showed that:

- The amount of NAT protein did not differ in the two genotypes, using Western blots.
  ➔ Confirming that there is no difference in uptake by the NAT in the two genotypes.
- Systemic injection of RX 821002 increases PFC NA efflux in NK1R+/+, but not NK1R-/-.
  ➔ Suggesting that function of the α_2-adrenoceptors *on the cell bodies in the LC* is disrupted in NK1R-/- mice (*cf.* Herpfer *et al* (2005)).
- [^{35}S]GTPγS binding of α_2-adrenoceptors (the α_{2A}-subtype) was ~ 70% lower in the LC of NK1R-/- than NK1R+/+ mice, but there was no difference in the PFC.
  ➔ Confirming that, in NK1R-/- mice, desensitization of α_2-adrenoceptors occurs to those located on the cell bodies in the LC, but not those on the terminals in the PFC.

Taken together, these findings indicated that the greater basal NA efflux in anaesthetized NK1R-/- mice is caused by ***increased transmitter release***, which is coupled with ***desensitization of somatodendritic α_{2A}-adrenoceptors*** (see Fig 1.5). However, it is not known whether receptor desensitization is the cause for, or the result of, the increased release in the mutants. It is not yet

clear why, in NK1R-/- mice, functional impairments occur to α_2-adrenoceptors in the LC, only. This echoes the finding of desensitization of 5-HT_{1A} receptors in the DRN, but not the PFC in these mutants (Froger *et al*, 2001). There is evidence that somatodendritic α_2-adrenoceptors in the LC differ from the cortical terminal ones: 1). Receptor reserve is larger in the LC than in the PFC (Pineda *et al*, 1997; Agneter *et al*, 1993); 2). Their susceptibilities for desensitization are different, based on the microdialysis evidence that long-term antidepressant treatment leads to desensitization of α_2-adrenoceptors regulating the local release of NA in both the LC and the cortex, but not those modulating the LC NA firing activity (Mateo *et al*, 2001). It is possible that NA has a greater affinity for the somatodendritic autoreceptors than for the terminal ones, hence desensitization occurs only to the former receptors in the mutants.

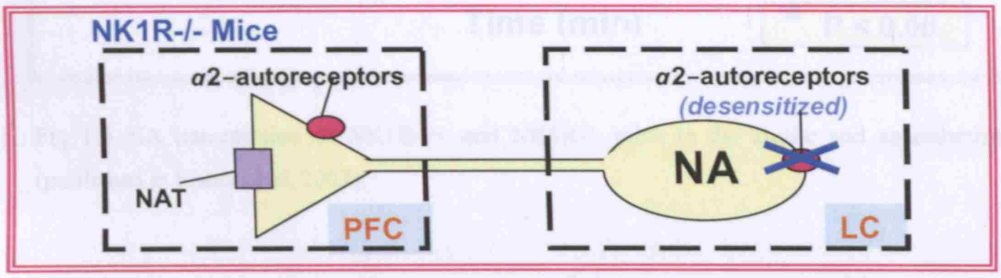


Fig 1.5 Impaired regulation of NA release by somatodendritic α_2-autoreceptors in NK1R-/- mice.

In contrast to the data obtained in anaesthetized mice, there is no difference in baseline NA efflux in freely-moving NK1R+/+ and NK1R-/- mice (Fisher *et al*, 2007). To explain this discrepancy, baseline NA efflux both before and after induction of anaesthesia with halothane was monitored, using *in vivo* microdialysis (Fisher *et al*, 2007). The results showed that NA efflux was reduced by halothane in both genotypes, but this reduction is greater in NK1R+/+ than in NK1R-/- mice (see Fig 1.6). It is well known that there are synergistic interactions between anaesthetics and α_2-adrenoceptors: α_2-adrenoceptor agonists increase the depth of anaesthesia (Bloor & Flacke, 1982) and halothane anaesthesia blunt NA release (Chave *et al*, 1996), most likely by potentiating the α_2-adrenoceptor-mediated reduction in LC neuronal firing (Saunier *et al*, 1993). Increased feedback inhibition by α_{2A}-adrenoceptors under anaesthesia would then explain the decrease in NA efflux in anaesthetized wild-types. Since the α_{2A}-adrenoceptors in the LC are desensitized in NK1R-/- mice, the inhibitory effect of halothane on NA release is less pronounced in these mice. This might therefore explain why NA efflux in NK1R-/- mice is apparently greater than in

NK1R+/+ mice under anaesthesia (Fisher *et al*, 2007; see Fig 1.6). However, in awake animals, this genotype difference might have been masked by reuptake processes.

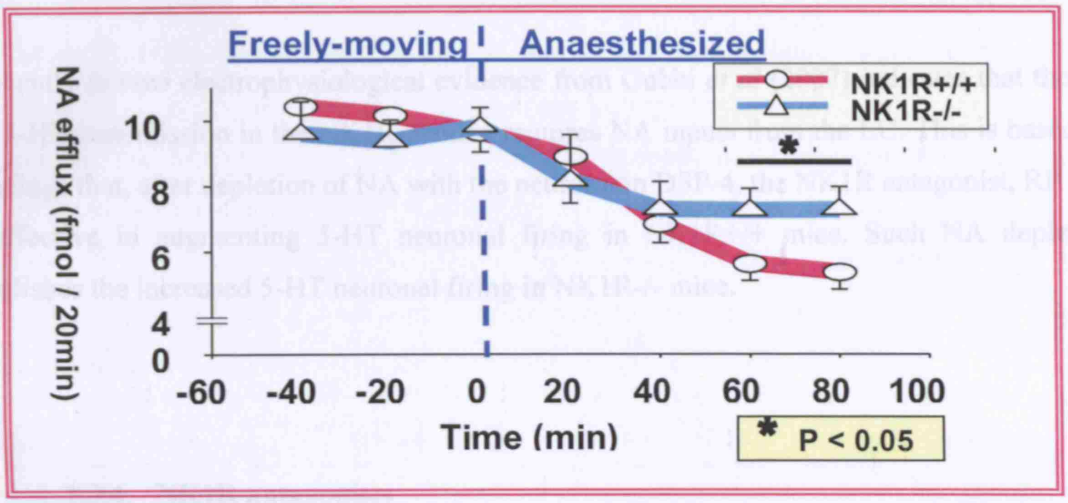


Fig 1.6 NA transmission of NK1R+/+ and NK1R-/- mice in the awake and anaesthetized states (published in Fisher *et al*, 2007).

1.3.3.3. Abnormal 5-HT transmission in the brain of NK1R-/- mice

Other abnormalities of NK1R-/- mice include impaired regulation of central serotonergic system, as revealed by Froger *et al* (2001):

- *In vivo* Microdialysis:

There was no genotype difference in basal 5-HT efflux in the PFC, but the increase in cortical 5-HT efflux following systemic injection of the selective serotonin reuptake inhibitor, paroxetine, is 4 ~ 6 fold higher in freely-moving NK1R-/- than in NK1R+/+ mice.

- Autoradiographic labeling:

At the level of the DRN, NK1R-/- mice showed a *reduction* in (i) 5-HT_{1A}-receptors labeling by the selective antagonist radioligand, [^{3}H]WAY 100635; (ii) 5-HT_{1A}-dependent [^{35}S]GTP-γ-S binding.

- Measurement of 5-HT_{1A}-receptor mRNA

A lower expression (-19 to -46%) of the receptor mRNA was found in NK1R-/- mice

Therefore, these findings suggest that regulation of 5-HT transmission in the PFC of NK1R-/- mice is impaired, which is most likely due to downregulation / desensitization of 5-HT_{1A}-autoreceptors in the DRN.

Recently *in vivo* electrophysiological evidence from Gobbi *et al* (2007) indicates that the increase in 5-HT transmission in the NK1R-/- mice requires NA inputs from the LC. This is based on their findings that, after depletion of NA with the neurotoxin DSP-4, the NK1R antagonist, RP 67580, is ineffective in augmenting 5-HT neuronal firing in NK1R+/+ mice. Such NA depletion also abolishes the increased 5-HT neuronal firing in NK1R-/- mice.

1.3.4. NK1R antagonists

It must be borne in mind that genetic modifications, such as null-mutation, could lead to developmental / compensatory change(s). When a genotype difference is observed in NK1R+/+ and NK1R-/- mice, it would be important to ascertain whether this is attributed to a lack of functional NK1 receptors, or to any developmental / adaptive change(s), in the mutants. To verify this, selective NK1R antagonists could be administered in both genotypes.

When choosing an antagonist, three factors should be considered: (1) penetration through the blood-brain barrier (BBB), (2) species variations in pharmacology / potency (Fong *et al*, 1992), and (3) nonspecific pharmacological effects of NK1R antagonists (see Table 1.3).

Based on the aforementioned three criteria, GR 205171 ($pK_i = 9.5$ in the rat cortex: Gardner *et al*, 1996), is thought to be the best NK1R antagonist for the current study. However, distribution of this drug for research has been blocked by the manufacturer, GlaxoSmithKline (GSK). Therefore, the NK1R antagonist, RP 67580 was used instead, which is also suitable for the investigation of the rat / mouse NK1 receptor, based on its higher affinity in these species than in humans (see Table 1.3).

To control for any nonspecific effects of RP 67580, another NK1R antagonist, L 733060, was used in the current study, despite its higher affinity for the human NK1 receptor (Rupniak *et al*, 2000). This is because there is microdialysis evidence that L 733060 is effective in NK1R+/+ mice (Guiard *et al*, 2004; also see Table 1.3).

In this project, the use of NK1R-/- mice removes the need to use the less potent enantiomer of RP 67580 or L 733060 to check for selectivity.

NK1R Antagonists	BBB Penetration	Species with Greater Potency	Inactive Enantiomer	References
CP-96,345	Good	Human /Guinea-pig / Gerbil	CP-96,344	Fong *et al*, 1992
CP-99,994	Good	Human / Guinea-pig / Gerbil	CP 100263	Tattersall *et al*, 1994
FK 888	Good	Human /Guinea-pig / Gerbil	N / A	Aramori *et al*, 1994
GR 205171	Good	Rat / Mouse	GR 226206	Gardner *et al*, 1996; Millan *et al*, 2001
L 733060 *	Good	Rat / Mouse Human / Guinea-pig / Gerbil	L-733,061	Bang *et al*, 2003; 2004; Guiard *et al*, 2004; Harrison *et al*, 1994; Kramer *et al*, 1998
L 743310	Poor	Human /Guinea-pig / Gerbil	N / A	Tattersall *et al*, 1996
MK 869	Good	Human /Guinea-pig / Gerbil	N / A	Kramer *et al*, 1998
RP 67580 *	Good	Rat / Mouse	RP 68651	Fong *et al*, 1992; Garret *et al*, 1991; Tattersall *et al*, 1996
SR 140333	Poor	Rat / Mouse	N / A	Rupniak *et al*, 1997

Table 1.3 Comparison of various selective NK1R antagonists. *: NK1R antagonists used in this project. *Ki values*: L 733060 = not published, RP 67580 = 3 nM for rats, 56 nM for humans (Tattersall *et al*, 1996). IC_{50} values for rats: L 733060 = 550 nM, RP 67580 = 5 nM (Seabrook *et al*, 1996).

1.4. ADHD

ADHD, which affects 3 ~ 8% of school-age children in the UK and US (Taylor *et al*, 1991; Szatmari *et al*, 1992), is one of the most commonly diagnosed neuropsychiatric disorders in children, although symptoms can persist into adulthood (see Wilens *et al*, 2002). Patients with ADHD suffer from inattentiveness, hyperactivity and impulsivity. Data from retrospective and follow-up studies revealed that these core symptoms often co-occur with other psychiatric conditions, such as depression / anxiety and substance abuse (Biederman *et al*, 1996; see Fone & Nutt, 2005). Hence, ADHD has been considered as a major clinical and public health problem.

1.4.1. Subtypes of ADHD and diagnostic criteria

The three core symptoms of ADHD, namely inattentiveness, impulsivity and hyperactivity, might not occur simultaneously. According to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV), three subtypes of ADHD have been identified: inattentive, hyperactive-impulsive and combined (American Psychiatric Association, 1994; see Wilens *et al*, 2002).

- Inattentive subtype: used to be diagnosed as Attention Deficit Disorder (ADD) without hyperactivity. Affected individuals of this type may have more difficulties in school, but not manifest difficulties with friends or family.
- Hyperactive-impulsive subtype: these patients may do relatively well in school, but have difficulties at home or in situations of less guidance and structure.
- Combined subtype: has a combination of the above symptoms. This is the most commonly represented subtype, accounting 50 ~ 75% of all ADHD individuals (Paternite *et al*, 1995; Morgan *et al*, 1997).

Despite the differences, patients with these subtypes all score lower than non-ADHD controls on measures of academic and social performance, and they do not differ from one another on any of these dimensions (see Wilens *et al*, 2002).

1.4.2. Treatments of ADHD

ADHD is commonly treated with **psychostimulants** (e.g. *d*-AMP: Dexedrine, Adderall; MPH: Ritalin, Concerta, Metadate), **NAT blockers** (e.g. atomoxetine: Strattera) and **α_2-adrenoceptor agonists** (e.g. clonidine: Catapress; guanfacine: Tenex). These drugs are used on the basis of their extensive efficacy and safety data (see Greenhill & Osman, 1999; Wilens *et al*, 2002).

1.4.2.1. The actions of psychostimulants on central monoamine transmission

Both *d*-AMP and MPH (see Fig 1.7 for chemical structures) exert their stimulating effects by enhancing monoamine transmission. However, the mechanism under which such action is carried out differs in the two drugs.

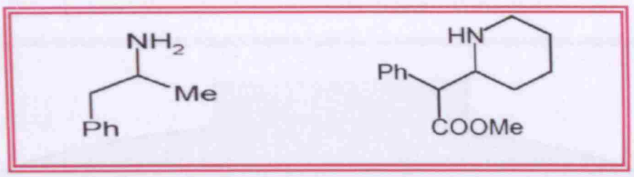


Fig. 1.7 The 2D structure of *d*-AMP (left) and MPH

***d*-AMP** augments extracellular concentrations of monoamines mainly by inhibiting their uptake and inducing impulse-independent release:

Competitive blockade of uptake

d-AMP is a substrate for the membrane-bound NAT. Hence it competes with extracellular NA for the uptake site, and is transported at the same time as Na^+ and Cl^- ions (see Seiden *et al*, 1993; Blakely *et al*, 1994; see Fig 1.8).

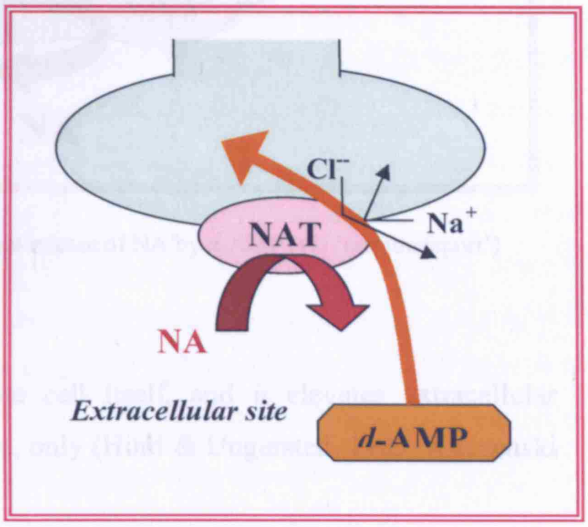


Fig 1.8 Diagrammatic illustration of uptake inhibition by *d*-AMP

Impulse-independent release

Once in the cytoplasm of the terminal, *d*-AMP is taken into the storage vesicles by the vesicular monoamine transporter, VMAT2. These vesicular transporters act as electrogenic antiporters (exchangers) of protons and monoamines, using a proton (or pH-driven) electrochemical gradient. As a weak base, *d*-AMP binds to a proton in the vesicle. The compound then has no charge, and can thus diffuse across the intravesicular membrane back to the cytoplasm. This would raise the pH of intracellular vesicles, consequently dissipating the electrochemical gradient across the intravesicular membrane, which drives transmitter uptake (by VMAT2) into the stores. As a result, deprotonated monoamines leak out of the vesicle into the cytoplasm, also. The cytoplasmic monoamines are then transported out of the cell by membrane-bound transporters. *Via* this reversal of uptake carriers, monoamines are 'released' into the synapse. This impulse-independent release process is known as 'retrotransport' (Sulzer & Rayport, 1990; Seiden *et al*, 1993; see Fig 1.9).

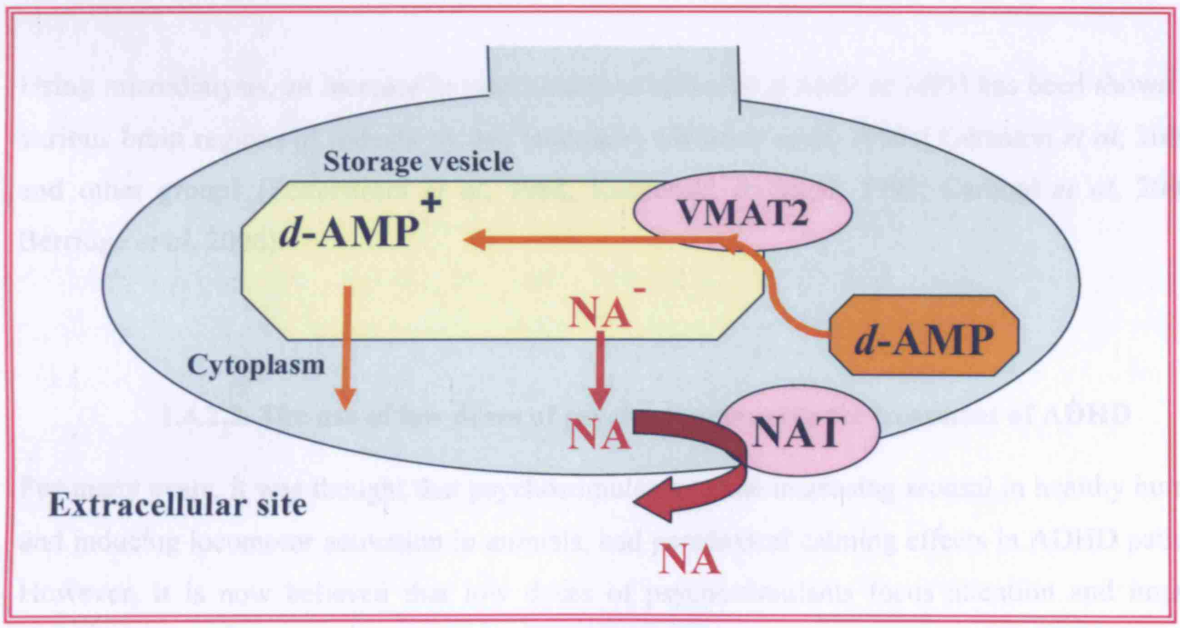


Fig 1.9 Diagrammatic illustration of impulse-independent release of NA by *d*-AMP (*via* 'retrotransport')

Unlike *d*-AMP, **MPH** is not transported into the cell itself, and it elevates extracellular concentrations of monoamines by blocking reuptake, only (Hurd & Ungersted, 1989; Kuczenski & Segal, 1997).

The affinity of the two psychostimulants for monoamine transporters differs. Specifically, *d*-AMP targets the NAT and the DAT with similar affinities, and is less potent at the serotonin transporter (SERT). In contrast, MPH has greatest affinity at the DAT, and it does not bind to the SERT (see Table 1.4).

Drugs	DAT	NAT	SERT	Species	References
d-AMP	34	38.9	3830	Rats	Rothman & Bauman, 2003
	41	23.2	11000	Humans	Eshleman *et al*, 1999
MPH	84	514	> 50000	Rats	*Gatley *et al*, 1996
	34	339	> 10000	Humans	Bymaster *et al*, 2002

Table 1.4 Affinities of *d*-AMP and MPH for DAT, NAT and SERT. *: Results from this study are IC_{50} values in nM. Results from the rest of the studies are *Ki* values in nM.

Using microdialysis, an increase in catecholamine efflux by *d*-AMP or MPH has been shown in various brain regions of rodents by this laboratory (Wortley *et al*, 1999a; Géranton *et al*, 2003) and other groups (Zetterstrom *et al*, 1988; Kuczenski & Segal, 1997; Carboni *et al*, 2003; Berridge *et al*, 2006).

1.4.2.2. The use of low doses of psychostimulants in the treatment of ADHD

For many years, it was thought that psychostimulants, while increasing arousal in healthy humans and inducing locomotor activation in animals, had paradoxical calming effects in ADHD patients. However, it is now believed that low doses of psychostimulants focus attention and improve executive functions in *both* normal and ADHD subjects, as revealed by the landmark studies from Kuczenski & Segal (2002, 2005). These authors found that, in adolescent rats, low oral doses of MPH—doses that produce blood levels similar to those observed in children taking stimulants to treat ADHD symptoms—in fact caused: (i) little or no change in DA efflux in the NAc and (ii) no locomotor activation. Hence, it was not that psychostimulants had paradoxical effects, but rather that rats had previously been given doses which exceed those used in the clinic.

In the current project, *d*-AMP and MPH have been injected intraperitoneally at a dose of 2.5 mg/kg, which is a moderate dose for mice. For the current project, this dose was chosen, rather than a lower one (such as 0.5 mg/kg for *d*-AMP: Kuczenski & Segal, 2001), because we did not set out to study ADHD. Instead, we intended to find a drug dose that produces a clear behavioural or neurochemical effect in the mouse brain. Various studies indicated 2.5 mg/kg (i.p.) as an effective dose for *d*-AMP (Huotari *et al*, 2004; Ganea *et al*, 2007) and MPH (Zhu *et al*, 2006). Hence, this dose was used in this project.

1.4.2.3. The use of α_2-adrenoceptor agonists in the treatment of ADHD

Clinical trials indicate that α_2-adrenoceptor agonists, such as clonidine, improve attention and decrease impulsivity in children with ADHD (Hunt *et al*, 1985). Nevertheless, the serious hypotensive and sedative side effects of these drugs make them undesirable as first-line medications. Recently, the use of the α_2-adrenoceptor agonist, guanfacine (Intuniv), in the treatment of ADHD has been submitted for approval by the Food and Drugs Administration (FDA). If approved, this drug would be the first α_2-adrenoceptor agonist used in clinic.

To date, the site where α_2-adrenoceptor agonists exert their therapeutic effects is still not clear. α_2-Adrenoceptors are present on both pre- and postsynaptic sites. Presynaptically, α_2-adrenoceptors are autoreceptors, which are located on NA cell bodies and terminals, regulating NA cell firing and release, respectively (see Section 1.2.2.1). Postsynaptically, α_2-adrenoceptors are heteroceptors found on non-NA cells, regulating release of other transmitters (e.g. DA: see Section 1.2.2.3).

Many researchers have emphasized the prevalence of presynaptic α_2-adrenoceptors in neuronal regulation (e.g. Svensson *et al*, 1975; see Starke *et al*, 1989; Starke, 2001; Simson & Weiss, 1989). However, Arnsten and colleagues argue that the site of action of α_2-agonists should be postsynaptic. This is based on their finding that the α_2-adrenoceptor agonist, clonidine, improves PFC cognitive performance in young monkeys, whose presynaptic element is destroyed, or depleted with 6-OHDA (Arnsten & Goldman-Rakic, 1985) or with the catecholamine-depleting agent, reserpine (Cai *et al*, 1993).

1.4.3. Neurobiology of ADHD

Owing to the high therapeutic value of psychostimulants and α_2-adrenoceptor agonists (although to a lesser extent) in treatment of ADHD, research on the neurobiology of this disorder has focused on catecholamine transmission in the brain, particularly in cortico-striatal circuits.

Genetic studies revealed that several catecholaminergic receptor polymorphic genes are linked with ADHD, including polymorphisms of the **D4** (Ebstein *et al*, 1997; LaHoste *et al*, 1996; Rowe *et al*, 1998; Swanson *et al*, 1998) and **D5 receptor** gene (Barr *et al*, 2000), the **DAT1** gene (Cook *et al*, 1995; Barr *et al*, 2001), and the genes encoding the NA synthesizing enzyme dopamine-β-hydroxylase (**DBH**) and for the **α_{2A}-adrenoceptor** (Roman *et al*, 2003; Park *et al*, 2005). Nevertheless, the effect size is small and none of these polymorphisms explains the complex symptoms of ADHD.

Theories regarding the involvement of central catecholaminergic systems in the pathophysiology of ADHD have concentrated on whether they are hypo- or hyper-functioning. Below are the examples of evidence supporting each hypothesis:

Hypo-DA transmission Hypothesis

- Neuroimaging studies, using functional magnetic resonance imaging (fMRI), showed that activation of the PFC is reduced (known as 'hypofrontality') in individuals with ADHD (Rubia *et al*, 1999). This can be reversed by *d*-AMP (Daniel *et al*, 1991) or the direct DA receptor agonist apomorphine (Daniel *et al*, 1989), hence indicating a deficiency of DA transmission in the PFC of ADHD patients.
- By using single photon emission computed tomography (SPECT), Krause *et al* (2000) showed that DAT expression in the basal ganglia of ADHD patients are markedly increased. This can be relieved after treatment with MPH.

Hyper-DA transmission Hypothesis

- In the ADHD model, DAT-/- mice, basal DA efflux in the dorsal striatum is greater than that in the wild-type. This might explain the hyperactivity of these mutants (Giros *et al*, 1996; see Section 1.4.4.2 for more details).

Hypo-NA transmission Hypothesis

- Psychostimulants (which increase catecholamine transmission) and α_2-adrenoceptor agonists (if postsynaptic) are used to treat ADHD (see Section 1.4.2.1).

Hyper-NA transmission Hypothesis

- This theory is mainly supported by various neurochemical studies in the spontaneous hypertensive rat (SHR) (de Villiers *et al*, 1995; Russell *et al*, 2000b), currently the best validated model of ADHD. However, inconsistent findings have been reported by Heal *et al* (2007a) (see Section 1.4.4.1).

Reconciling these controversial observations, it is possible that *both* insufficient and excessive central catecholamine transmission lead to ADHD. This is not surprising, as optimal transmitter signaling is most beneficial for functioning of many brain regions, such as the PFC (see Stanford, 1993, 1996; Arnsten, 1997). Further, ADHD is a complex disorder, which could be associated with higher catecholaminergic transmission in one brain region, but lower in others. These possibilities are yet to be tested.

1.4.4. Current animal models of ADHD

Over the years, several animal models of ADHD have been proposed, which are used to gain more in-depth insight into this disorder and to assist in development of new therapeutic strategies. A good model should: (i) mimic characteristics of the disorder (face validity), (ii) conform with an underlying theory of this condition (construct validity) and (iii) enable predictions of future therapies (predictive validity).

The currently available models range from administration of neurotoxins to genetic modification models (see Table 1.6), all of which indicate abnormal central monoamine transmission (especially DA and NA). However, it is difficult to compare the neurochemical changes across these models, as studies vary greatly in terms of techniques and brain areas under investigation.

Here, the best validated rat and mouse models, the SHR and the DAT-/- mouse, are described.

1.4.4.1. The spontaneous hypertensive rat (SHR)

The SHR is so far the most widely studied model of ADHD. These animals were created by Okamoto and Aoki (1963), who inbred the Wistar–Kyoto (WKY) rat that exhibited high systolic blood pressure and stress reactivity. The SHR was later used as an ADHD model, after the discovery of their behavioural abnormalities which echo symptoms of this condition (Sagvolden *et al*, 1992; see Sagvolden, 2000).

Face Validity

The SHR are **inattentive** (seen as increased percentage of errors in operant tasks) and **hyperactive** (which is not present in novel, non-threatening situations, but develops over time when reinforcers are infrequent), compared with their comparator, the WKY (Mook *et al*, 1993; Sagvolden *et al*, 1992, 1993, 1998, 2000). The SHR also shows **impulsivity**, as they are intolerant to delayed reward (see Sagvolden, 2000).

Construct Validity

There is a great deal of evidence that central DA and NA transmission in the SHR are disrupted, but data are inconsistent regarding whether the transmission is decreased (hypo-) or increased (hyper-) (see Table 1.5).

The disparities between the two general views could be, at least in part, due to the use of different comparators. The SHR was compared with the WKY in most studies. However, Heal and colleagues argued that the SD could be a better comparator because: (i) this strain is widely used in microdialysis studies investigating actions of ADHD medications (e.g. Sharp *et al*, 1987; Géranton *et al*, 2003a, b, 2004); (ii) the WKY shows atypical behavioural and neurochemical characteristics, which make them different from other common rat strains (Diana, 2002; Drolet *et al*, 2002) (personal communication with Prof. David Heal, see Heal *et al*, 2007).

Predictive Validity

The SHR and its controls (WKY or Sprague-Dawley rats) both showed an increase in locomotor activity after treatment with *d*-AMP (McCarty *et al*, 1980; Hynes *et al*, 1985; Tsai & Lin, 1988)

and MPH (Wultz *et al*, 1990; Amini *et al*, 2004; Yang *et al*, 2006) at low to moderate doses, although the increase in the SHR is smaller than in the controls. This therefore reduces the validity of the SHR as a model of ADHD.

	Theory 1	Theory 2
DA	**Hypo-DA** • Using an *in vitro* superfusion technique, slices of the PFC, NAc and dorsal striatum in the SHR were shown to release less [^{3}H]DA than in the WKY upon electrical and K^+-stimulation (Linthorst *et al*, 1990; Russell *et al*, 1995, 1998, 2000b). • Compared with the WKY, efflux of DA and its metabolite, DOPAC, were lower in the caudate nucleus of SHR than WKY (Linthorst *et al*, 1991). This could be associated with an increased DA D2 autoreceptor density in this brain region of the SHR (Russell *et al*, 1995; Russell, 2000a).	**Hyper-DA** • Basal DA efflux in the NAc shell is greater in the SHR than the WKY (Carboni *et al*, 2003). This might explain the hyperactivity of the former strain. • Basal DA efflux in the dorsal striatum was 78% higher in the SHR than Sprague-Dawley (SD) rats (Cheetham *et al*, 2007; Rowley *et al*, 2007; see Heal *et al*, 2007).
NA	**Hyper-NA** • Gene expression of the catecholamine-synthesizing enzyme, tyrosine hydroxylase, is higher in the ventrolateral medulla oblongata of SHR than WKY (Reja *et al*, 2002a), consistent with elevated NA efflux in several brain areas of SHR, including the LC, the SN and the PFC (de Villiers *et al*, 1995). • The SHR has lower expression of α_{2A}-adrenoceptor mRNA, hence less efficient auto-inhibition of NA release than the WKY (Russell *et al*, 2000b; Reja *et al*, 2002b). Similar finding was observed in NK1R-/- mice by our group (Herpfer *et al*, 2005; Fisher *et al*, 2007).	**Hypo-NA** • Basal NA efflux was 26% lower in the PFC in SHR than in SD (Cheetham *et al*, 2007; Rowley *et al*, 2007; see Heal *et al*, 2007).

Table 1.5 Inconsistent findings on the neurochemical changes in the SHR.

Finally, another potential confounding factor for the SHR as a model for ADHD is their hypertension. Many behavioral deficits in SHR, especially those related to learning and memory,

might reflect brain dysfunction or damage caused by high blood pressure. Moreover, it is not clear whether beneficial effects of drugs, such as the α_2-adrenoceptor agonists, clonidine and guanfacine, in this model reflect the direct central neuropharmacological effects of these agents, or their effects on lowering the blood pressure (see Sagvolden, 2000; Ferguson, 2001).

1.4.4.2. The DAT knockout mouse (DAT-/-)

Face Validity

The DAT-/- mice are **hyperactive**, which is exacerbated upon exposure to a novel environment (Giros *et al*, 1996; Gainetdinov *et al*, 1999). Moreover, these mutants have **poor behavioural inhibition**, as demonstrated in the eight-arm radial maze test (a standard approach to evaluate spatial cognitive function in rodents) (Gainetdinov & Caron, 2001).

Construct Validity

Due to the blunted clearance process by the DAT, there is a 5-fold increase in spontaneous DA efflux in the striatum of the DAT-/- than in the wild-type (Giros *et al*, 1996). This model therefore suggests an association between ADHD and hyper-DA transmission in the brain. The reduced DA reuptake causes several profound adaptive changes in the mutants. These include increased activity of the DA synthesizing enzyme, tyrosine hydroxylase (TH) (Giros *et al*, 1996), as well as downregulation of postsynaptic D1 and D2 receptors, as reflected by ~ 50% decrease in their mRNA and protein levels in the basal ganglia (Gainetdinov *et al*, 1998; Jaber *et al*, 1996, 1999).

Predictive validity

Hyperactivity of DAT-/- mice is attenuated by *d*-AMP and MPH (Jones *et al*, 1998b; Gainetdinov *et al*, 1998; Gainetdinov & Caron, 2001). It then raises the question of how the psychostimulants achieve this, given that the DAT is not functional in these animals.

Various possibilities have been proposed (see van der Kooij & Glennon, 2007). For example, psychostimulants might exert their calming effects in the DAT-/- mice by targeting other monoamine transporters (notably the NAT and SERT) which also take up extracellular DA

(Morón *et al*, 2002). The involvement of the NAT is supported by the finding that the behavioural responses of DAT-/- mice to *d*-AMP and cocaine are mimicked by selective inhibitors of the NAT, but not by inhibitors of the DAT (Carboni *et al*, 2001). Moreover, there is evidence that hyperactivity of the mutants can be blocked by 5-HT releasing agent fenfluramine, the SSRI fluoxetine, and the 5-HT agonist quipazine (Gainetdinov *et al*, 1999), suggesting the contribution of the central 5-HT system to the pharmacological effects of psychostimulants in the mutants.

Despite good face and predictive validities, this mouse model has several disadvantages. The DAT-/- mice display growth retardation (Bosse *et al*, 1997) and an increased risk of premature death (Giros *et al*, 1996; Gainetdinov *et al*, 1998) with the result that only 68% of the animals remain alive by 10 weeks of age. Moreover, they lack functional DATs, hence hampering the discovery of potential ADHD treatments which target the DAT.

	Animal model	**Face validity**			**Construct validity**			**Predictive validity**	
		Hyper-activity	**Inatten-tiveness**	**Impul-sivity**	**DA**	**NA**	**5-HT**	**MPH**	***d*-AMP**
RAT	Neonatal 6-OHDA lesioned rat	✓	✓	—	↓	↓	↓	✓	✓
	Neonatal hypoxia model	✓	—	—	↓	—	—	—	✓
	The prenatal BrdU model	✓	—	—	↓	—	↑	X	—
	Cerebellar stunting in rats	✓	—	—	—	—	—	X	X
	Hippocampal irradiation	✓	✓	—	—	—	—	—	✓
	SHR	✓	✓	✓	↓ or ↑	↑ or ↓	—	X	X
	NHE rat	✓	✓	—	↑	—	—	—	—

Continued on the next page.

	Animal model	Face validity			Construct validity			Predictive validity	
		Hyper-activity	Inatten-tiveness	Impul-sivity	DA	NA	5-HT	MPH	*d*-AMP
Mouse	DAT KO mouse model	✓	—	✓	↑	—	—	✓	✓
	DAT KD mouse model	✓	—	✓	↑	—	—	—	✓
	Coloboma mouse	✓	—	—	↓	↑	↓	X	✓
	Hyperactive wheel Running mouse	✓	—	—	—	—	—	✓	—
	Acallosal mouse	✓	✓	—	—	—	—	—	—
	TRbetaPV KI mice	✓	✓	✓	—	—	—	X	—
	NK1R-/- mice	✓	—	—	↓	↑ or ↓*	↑	✓	✓

Table 1.6 Validating ADHD models on behavioural parameters, affected neurochemistry and psychostimulant responses. '✓': this characteristic of ADHD is confirmed by the proposed animal model; 'X': this characteristic is in contrast with the proposed animal model; '—': this characteristic has not yet been investigated in the proposed animal model; ↑: hyper-functioning of the indicated monoamine system; ↓: hypo-functioning of the indicated monoamine system. Adapted and modified from van der Kooij & Glennon (2007). *: PFC NA efflux is greater in anaesthetized NK1R-/- mice than in wild-type, but it declined progressively in freely-moving mutants when animals were confined in the novel light zone of the light/dark exploration box (see Chapter 7).

1.5. Aim of the study

As described in this Chapter, NK1R-/- mice are hyperactive and have disrupted regulation of NA release by α_2-adrenoceptors. These abnormalities led to the investigation of the following issues:

- **Determine the neurochemical factors underlying the hyperactivity of NK1R-/- mice**
- **Compare the locomotor and catecholaminergic responses of NK1R+/+ and NK1R-/- mice to acute administration of the psychostimulants, *d*-AMP and MPH**
- **Test whether the behavioural and neurochemical abnormalities of NK1R-/- mice were due to a lack of functional NK1 receptors, or to developmental / compensatory changes**

1.6. Objectives

Chapter 3: To test the impulse-dependency of NA release by infusing high K^+-containing and / or Ca^{2+}-free Ringer's solution in the PFC of freely-moving NK1R+/+ and NK1R-/- mice.

Chapter 4: To compare the NA response to local infusion of *d*-AMP in the PFC in NK1R+/+ and NK1R-/- mice, and to investigate whether the genotype difference (if any) could be explained by a difference in function of α_2-adrenceptors. Further, the NA and DA response to local infusion of MPH in the PFC was also tested in the two genotypes.

Chapter 5: To profile the behaviour of NK1R+/+ and NK1R-/- mice in the LDEB in response to systemic administration of *d*-AMP and MPH. Whether or not the genotype differences (if any) were due to absence of functional NK1 receptors in NK1R-/- mice was examined using a selective NK1R antagonist, RP 67580 or L 733060.

The findings reported in Chapter 5 revealed a calming effect of d-AMP and MPH in NK1R-/- mice, suggesting these mutants could be a novel model of ADHD. Therefore, the rest of the project was carried out to test the validity of this model by investigating their DA and NA responses to d-AMP.

Chapter 6: To characterize the DA response in the PFC and the dorsal striatum to systemic administration of *d*-AMP in NK1R+/+ and NK1R-/- mice in the LDEB. The NK1R antagonist, RP 67580, was again used to test whether or not the genotype difference in DA transmission could be explained by disruption of NK1 receptors in the mutants.

Chapter 7: To characterize the PFC NA response to systemic administration of *d*-AMP in NK1R+/+ and NK1R-/- mice in the LDEB. Moreover, changes in PFC NA efflux in the two genotypes, after the treatment with a selective α_2-adrenoceptor antagonist, RX 821002, were also compared. The possibility that the genotype differences could be caused by a lack of functional NK1 receptors in NK1R-/- mice was again tested, using the NK1R antagonist RP 67580.

Chapter 2. Methods and Materials

2.1. Introduction

Over the past few decades, a variety of techniques has evolved to enable the investigation of neurotransmitter systems in the CNS and their involvement in any specific physiological and / or behavioural event. In this project, *in vivo* microdialysis and the light / dark exploration box paradigm have been used to monitor any neurochemical and behavioural changes of NK1R+/+ and NK1R-/- mice, in response to drug challenges. These two approaches are described in detail in this chapter.

2.2. Animals

2.2.1. Housing conditions

Mice were the only species used in this project.

Male adult mice (25 ~ 35 g) were obtained from a colony in Biological Services, University College London (UK). They were housed in groups of 1 ~ 5 per cage, with standard lab chow (Harlan Tekland TRM Rat / Mouse Diet; Harlan, Bicester, U.K.) and tap water fed *ad libitum*. Environmental conditions were applied, i.e. 12:12 h light / dark cycle (light on at 08:00 h), with temperature and humidity controlled at 20 ± 2°C and 45 ± 5%, respectively.

All experiments complied with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines. Local ethical approval was gained.

2.2.2. NK1 knockout mice

Mice were originally derived from homologous recombination of C57BL/6 blastocysts implanted with genetically manipulated 129/Sv stem cells, which contained targeted disruption of the NK1 receptor gene in exon 1 (De Felipe *et al*, 1998).

Exon 1 of the gene in 129/Sv stem cells was disrupted by insertion of a cassette into the unique *Stu*I site. The cassette consisted of an internal ribosome entry site (IRES) and the *lacZ* coding

sequence, together with a neomycin-resistance gene expressed from its own promoter. The targeted clones were then injected into C57BL/6 blastocysts, and chimaeric males were mated with C57BL/6 females. Mice homozygous for the NK1 mutation were then produced by crossing heterozygotes (see Fig 2.1; Diagram and legend adapted from De Felipe *et al*, 1998). The original NK1R-/- mice were then crossed onto an outbred MF1 strain (Harlan OLAC, Bicester, U.K.).

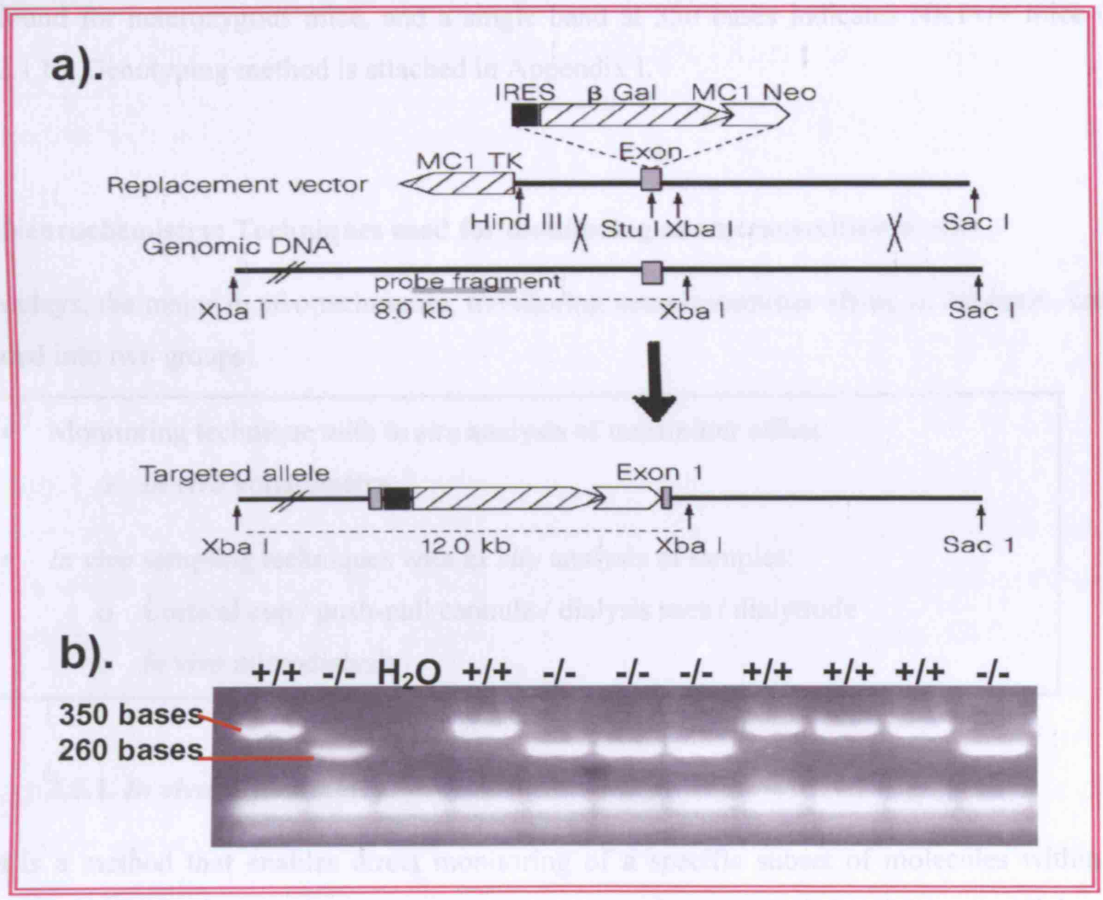


Fig 2.1. NK1 receptor disruption by homologous recombination. **a).** Demonstrating the region of the wild-type NK-1 locus containing exon 1, the targeting (replacement) vector and the predicted structure of the targeted NK-1 receptor gene. The 5' external probe used for Southern blot analysis and the sizes of the restriction fragments detected with this probe in the wild-type and targeted alleles are indicated (Diagram and legend obtained from De Felipe *et al*, 1998). **b).** genotyping by performing PCR on mouse tail DNA. A single 350-base band is shown for NK1R+/+ mice, and a single 260-base band is shown for NK1R-/- mice.

2.2.3. Genotype verification

After every experiment, postmortem verification of genotype was performed by running the polymerase chain reaction (PCR) on DNA isolated from tail tip samples. Three PCR primers were used in this process: NK1-F, NK1-R and NeoF. NK1-F and NK1-R amplify a 350-base section of exon 1 of the gene encoding the NK1 receptor, whereas NeoF and NK1-R amplify a 260-base section of the NK1 receptor gene including the inserted cassette. Therefore, on a 2% agarose gel, a single 260-base band is shown for NK1-/- mice, two bands at 260 and 350 bases are found for heterozygous mice, and a single band at 350 bases indicates NK1+/+ mice (see Fig 2.1 b). Genotyping method is attached in Appendix I.

2.3. Neurochemistry: Techniques used for monitoring neurotransmitter *in vivo*

Nowadays, the major *in vivo* techniques, monitoring neurotransmitter efflux in the brain, can be divided into two groups:

- Monitoring technique with *in situ* analysis of transmitter efflux:
  - *In vivo* voltammetry
- *In vivo* sampling techniques with *ex situ* analysis of samples:
  - Cortical cup / push-pull cannula / dialysis sacs / dialytrode
  - *In vivo* microdialysis

2.3.1. *In vivo* voltammetry

This is a method that enables direct monitoring of a specific subset of molecules within the extracellular fluid of brain (Adams *et al*, 1978; see Stamford, 1989). It involves an oxidizing or a reducing electrode, which is a carbon-fibre electrode of 5-30 μm in diameter implanted in the experimental tissue. Oxidation of solutes on the surface of the electrode then generates the Faradaic current, which is proportional to the solute concentration. Relative to microdialysis, i*n vivo* voltammetry offers a better spatial resolution (due to smaller diameter of the microelectrodes) and temporal resolution (real-time monitoring with fast cyclic voltammetry and chronoamperometry). However, this approach is mainly limited to the study of monoamines and

their metabolites, as it can only measure electroactive species with good diffusion properties. Moreover, it lacks the sensitivity and ability to separate and quantitate numerous compounds that exist simultaneously in the extracellular space of the brain, e.g. ascorbate.

2.3.2. Cortical cup / push-pull cannula / dialysis sacs / dialytrode

Over the past 60 years, development of different biochemical methods has permitted measurement of synthesis and clearance rates of neurotransmitters, including NA, DA and 5-HT.

Cortical cup

The cortical cup, developed by MacIntosh and Oborin in 1953, was the first *in vivo* preparation for sampling the extracellular environment in the CNS of unanaesthetized animals. With this method, a cylinder, filled with a perfusion fluid (aCSF or Ringer's solution), was placed in tight contact with the cortical surface of interest. The fluid collected by continuous flow or sequential washes was analyzed *ex situ*. However, this method had limited applications because it could only be used in the cortical surface and the sampling was discontinuous.

Push-pull cannula

To overcome the drawbacks of the cortical cup, another sampling method, the push-pull cannula, was developed by Gaddum in 1961. This method provided the first opportunity to continuously evaluate neurochemical events in discrete structures deep within the intact CNS. Generally, push-pull cannula consisted of two concentric tubes: inlet and outlet. Two pumps were required, working in synchrony as pushing and pulling devices: one to force the perfusion fluid down and the other one to pull up. Nevertheless, the problem with this sampling tool was tissue perturbation caused by directly infusing a perfusion solution into the brain area of interest as well as by high perfusion rates (typically 150 µl/min).

Dialysis sacs

Based on the theory of the push-pull cannula technique, a modified method was proposed. In 1966, Bito and colleagues determined brain amino acid levels by implanting dialysis sacs into the cerebral hemispheres of dogs, which were removed several days later. This technique circumvented the problems associated with having a perfusion solution coming into direct contact with brain tissue, but the sampling was discontinuous.

Dialytrode

In 1972, Delgado et al developed the dialytrode, a push-pull cannula with a small permeable bag attached to the end. This enabled continuous sampling of the extracellular environment.

2.3.3. *In vivo* microdialysis

These early attempts (see above) laid the foundation for subsequent work in dialyzing extracellular fluid of brain. In 1974, Ungerstedt and Pycock published the first modern microdialysis set-up. Since then, this technique has become widely used to monitor changes in neurotransmission (see Ungerstedt, 1984; Di Chiara, 1990; Benveniste & Hüttemeier, 1990; Sharp & Zetterstrom, 1992; Chaurasia, 1999). It is noteworthy that *in vivo* microdialysis does not measure release of solutes, but monitors *changes* in extracellular concentration ('efflux') of solutes. This technique bears various advantages as well as disadvantages, which have all been listed in Table 2.1.

Microdialysis itself is only a sampling technique. The ability to measure transmitters in the dialysate depends entirely on the sensitivity of an appropriate analytical method, such as high performance liquid chromatography (HPLC; see Section 2.3.5).

Pros	• Able to collect low molecular weight compounds (5~50 kDa) from remote brain regions with minimal perturbation to the system under study (*cf.* earlier techniques). • Dialysate collection is continuous. • Can be used in both freely-moving and anaesthetized animals. • Suited to examination of long lasting changes in the content of extracellular fluid (samples collected at 5~20 min intervals).
Cons	• It is an invasive technique and it causes inevitable neuronal damage. • Poor time resolution. • Probe recovery may under-estimate the actual extracellular concentration of substance of interest.

Table 2.1 Advantages and disadvantages of *in vivo* microdialysis

2.3.4. Principle and procedure of *in vivo* microdialysis

Brain microdialysis involves inserting a microdialysis probe into a selected brain area of a living animal under anaesthesia. After the surgery, a perfusion solution is slowly pumped through the microdialysis probe, during which time the exchange of molecules in the perfusate and those in the extracellular fluid of the tissue occurs. Dialysates are collected at regular time intervals and are analyzed, *ex situ*, using high performance liquid chromatography (HPLC) coupled to electrochemical detector (ECD).

2.3.4.1. The microdialysis probe

- **Probe design**

There are four principal types of microdialysis probes (see Fig 2.2):

A). The linear probe (Imperato & Di Chiara, 1984)
  - Consists of a hollow fibre microdialysis membrane connected to small bore tubings on both ends.
  - Flexible and useful for implantation in peripheral tissue (e.g. the heart).

While the linear probe requires two points of entry, the other three designs only require one (in- and outlet tubes are positioned in a parallel fashion):

B). The loop probe (Zetterstom *et al*, 1982):
  - Consists of a long folded dialysis membrane connecting two cannulae.
  - It was used in the brain, but caused huge lesions. It is now mainly used for subcutaneous tissue or peritoneal sampling. The increased surface area augments the yield of transmitter contents in the dialysate.

C). The side by side probe (Sandberg *et al*, 1986):
  - A modification of the concentric probe (see below), where fused silica tubes attached side-by-side to each other and covered with a cylindrical dialysis membrane sealed at the tip.

- Suitable for intravenous sampling.

D). The concentric probe (Tossman & Ungerstedt, 1986):

- Made of a cylindrical piece of dialysis membrane sealed with an adhesive at one end, the other being attached to a solid metal cannula consisting of an inner and outer tubing.
- Most commonly used for *in vivo* microdialysis of brain, vasculature and systemic tissues.

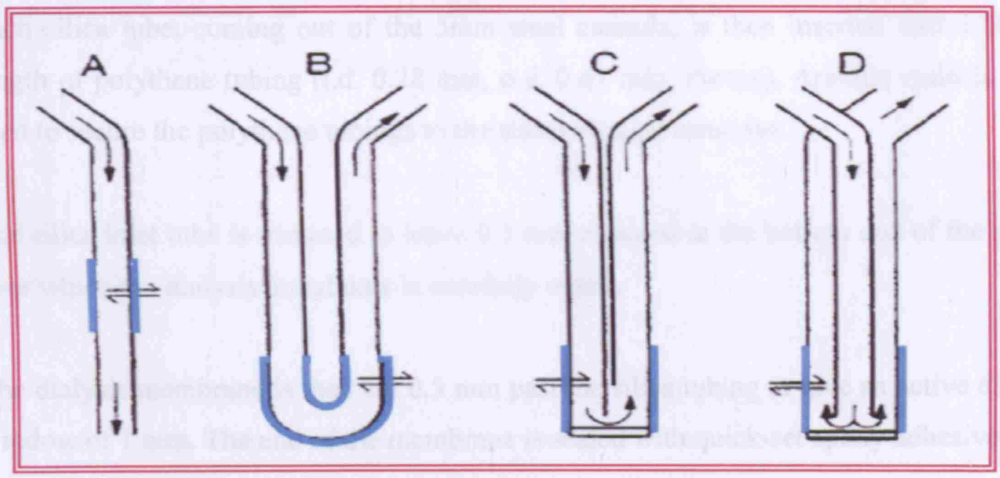


Fig 2.2 Schematic illustration of four basic types of microdialysis probes: **A)** linear, **B)** loop, **C)** side-by-side, and **D)** concentric. The blue area represents the dialysis membrane. Arrows refer to direction of flow of perfusate through probes. Diagram obtained from Gunaratna *et al* (1994).

- **Probe construction**

Microdialysis probes used in this project were of a side-by-side design and were made in-house. The details of probe construction are as follows (the step-by-step process of probe preparation is shown in Fig 2.3):

1. Two silica capillary tubes (i.d. 75 μm; o.d. 150 μm, Scientific Glass Engineering, UK), cut to a length of 40 mm with a scalpel blade, are inserted side-by-side into a 15 mm stainless steel cannula (i.d. 380 μm, o.d. 500 μm, Goodfellow Cambridge Ltd).

2. A 5mm steel cannula is attached over each silica tube (top end of probe) to produce a fork-shaped frame. Thus, the two short cannulae eventually become the inlet and outlet of the probe. A drop of quick-set epoxy adhesive (Araldite) is applied to seal the junction of the main cannula and the two short cannulae.

3. Each silica tube, coming out of the 5mm steel cannula, is then inserted into a 50 mm length of polythene tubing (i.d. 0.28 mm, o.d. 0.61 mm, Portex). Araldite resin is again used to secure the polythene tubings to the stainless steel cannulae.

4. The silica inlet tube is trimmed to leave 0.5 mm exposed at the bottom end of the probe, over which the dialysis membrane is carefully eased.

5. The dialysis membrane is then cut 0.5 mm past the silica tubing to give an active dialysis window of 1 mm. The end of the membrane is sealed with quick-set epoxy adhesive (RS)

After construction, probes were kept in an airtight container until use.

The dialysis membrane, which forms the interface for the transfer of substances between the perfusion medium and the extracellular fluid, is a critical component of the microdialysis probe. The pore size of the membrane should be optimal, so that it is large enough for free diffusion of solute molecules, but small enough to exclude macromolecules (e.g. proteins). Dialysis membranes can be made of various materials, such as cellulose (Dow), polycarbonate (Carnegie) and acrylic copolymer (Amicon Vitafibre), polyacrilonitrile copolymer (AN.69-HOSPAL). These membranes differ in molecular weight cut-off (5~50 kDa) and permeability to solutes (see Di Chiara, 1990). In this project, all microdialysis probes were made with cuprophan, a regenerated cellulose membrane (Medicell International Ltd, UK; molecular cut-off: 5 kDa; i.d. 0.20 mm, o.d. 0.25 mm). This is because this type of membrane is biocompatible, i.e. it does not

interact with any neurotransmitter, or have any immunologic (allergic / inflammatory) and / or toxic effects.

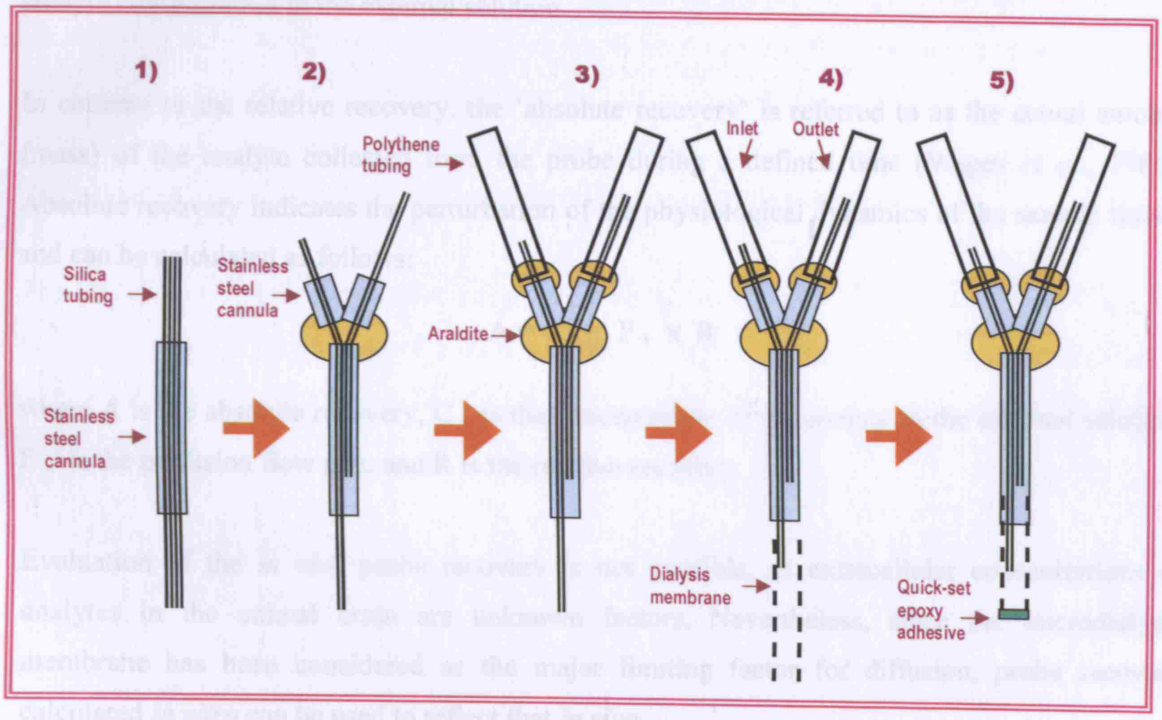


Fig 2.3 Illustration showing step-by-step construction of the microdialysis probes used in this project.

- **Probe efficiency (probe recovery)**

During microdialysis sampling, analytes are removed from the extracellular space via diffusion across the dialysis membrane. Therefore, the continuous flow of perfusion fluid through the probe does not allow the establishment of a steady-state equilibrium between the analyte concentrations inside and outside the probe. Under these non-equilibrium conditions, the analyte concentration in the dialysate is less than the actual concentration in the extracellular fluid around the probe. Therefore, before commencing the microdialysis studies, it is important to assess the performance of the probe by evaluating its relative recovery, which is the ratio of the fraction recovered to the actual concentration (Wages *et al*, 1986; Riley *et al*, 1994):

$$R = C_{p,f} / C_{e,f}$$

where R is the relative recovery, $C_{p,f}$ is the analyte concentration in the dialysate, and $C_{e,f}$ is the analyte concentration in the external solution.

In contrast to the relative recovery, the 'absolute recovery' is referred to as the actual amount (mass) of the analyte collected from the probe during a defined time (Wages *et al.*, 1986). Absolute recovery indicates the perturbation of the physiological dynamics of the sample tissue, and can be calculated as follows:

$$A = C_e \times F_v \times R$$

where A is the absolute recovery, C_e is the concentration of the analyte in the external solution, F_v is the perfusion flow rate, and R is the relative recovery.

Evaluation of the *in vivo* probe recovery is not possible, as extracellular concentrations of analytes in the animal brain are unknown factors. Nevertheless, since the microdialysis membrane has been considered as the major limiting factor for diffusion, probe recovery calculated *in vitro* can be used to reflect that *in vivo*.

Before assessing the *in vitro* probe recovery, several factors that influence it need to be considered:

- Flow rate of the perfusion fluid
- Membrane material
- Membrane surface area
- Composition of the perfusion medium

- **Flow rate of the perfusion fluid**

In microdialysis sampling, the concentration gradient of an analyte across the dialysate membrane is driven by the flow rate of the perfusion solution, hence flow rate determines the probe recovery. When the flow rate is lower, the analyte across the membrane will be closer to equilibrium. As a result, the relative recovery will approach 100% as the flow rate reaches zero, but decreases exponentially with increasing flow rate. Conversely, the absolute recovery is zero at zero flow rate, but increases as the flow rate goes up. It then plateaus off at higher flow rate, when no more substance can be delivered from the tissue to the perfusion medium.

The dialysis probe was perfused at 1.5 μl / min in this study. This is because this flow rate has been confirmed, by previous colleagues in this lab, as the optimal rate for achieving greatest possible recovery without dramatically decreasing the concentration gradient across the dialysis membrane.

- **Membrane material**

Details see before (see Section 2.3.4.1).

- **Membrane surface area**

A larger surface area of the dialysis membrane will give rise to a greater area available for exchange of molecules. Increasing the membrane area is normally achieved by increasing the dialysis window (i.e. the length of membrane fitted to the end of the probe). However, this can lower the spatial resolution and cause greater tissue damage. In this project, the dialysis window was 1 mm.

- **Composition of the perfusion medium**

Any differences in composition between the perfusion medium used and the extracellular fluid can cause additional concentration gradients between the two compartments, thus interfering with the recovery of the substance concerned. Therefore, the perfusion medium should mimic the brain extracellular fluid in terms of composition and pH, in order to minimize osmotic pressure effects and loss of substances from either the perfusion fluid or the extracellular fluid (Benveniste & Huttemeier, 1990). Artificial cerebrospinal fluid (aCSF) and Ringer's solution are

the two perfusion media normally adopted in microdialysis experiments. In the current project, the modified Ringer's solution was used (components listed below). This is because this solution, buffered by extracellular proteins and ions, contains essential ions that maintain ionic balance and, unlike aCSF, it does not contain additional ions (e.g. PO_4^{2-}) which do not normally exist in the brain.

- KCl 4 mM
- NaCl 145 mM
- $CaCl_2$ 1.3 mM

} Dissolved in deionized water (pH 6.8)

Assessment of the *in vitro* probe recovery

After considering the above factors that influence probe recovery, *in vitro* probe recovery was tested. An Eppendorf tube, containing 60 fmol NA standard solution, was place on ice, so as to prevent rapid decomposition of NA. A microdialysis probe was placed in this NA standard solution and was perfused with the modified Ringer's solution at the same flow rate as that used in the experiment (i.e. 1.5 µl/min). Samples were taken at 20 min intervals, followed by immediate analysis of the NA content in the dialysate using HPLC-ECD (see Section 2.3.5). In the dialysate, NA concentration was 7.57 fmol / 20 min, therefore the relative probe recovery was 12.6%:

$$R = C_{p,f} / C_{e,f}$$
$$= 7.57 / 60$$
$$= 0.126 \quad \text{(i.e. 12.6\%)}$$

Nevertheless, measurements in this project were not corrected for probe recovery. This is because this study aimed to investigate *relative* changes in brain catecholamine efflux in response to various CNS drugs. Hence, any drug-induced changes were compared against the basal efflux. In addition, colleagues in this lab have previously found that the recovery was constant between probes and over the range of catecholamine concentrations generally measured.

2.3.4.2. Surgical procedure

- **Induction of anaesthesia**

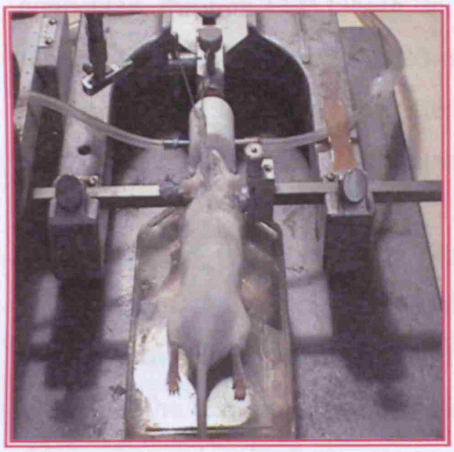
Anaesthesia of mice was induced in an enclosed transparent chamber (in cm: 12.5 x 13.5 x 26.5), which delivered inhalational anaesthetics (2% halothane (Pfizer, UK) combined with 95% O_2 / 5% CO_2 at 2 L/min). A couple of minutes later, the righting reflex was tested to ensure sufficient depth of anaesthesia, before progressing with surgery. Upon the loss of the righting reflex, mice were quickly transferred from the induction chamber to a stereotaxic frame (Kopf). Anaesthesia was then maintained by the delivery of 1.8% halothane mixed with 95% O_2 / 5% CO_2, at 2 L/min, *via* a face-mask.

- **Probe insertion**

Mice were secured in a flat-skull position by using the incisor and blunt ear bars. The midline of the scalp was slit with a sterile blade, and the surface of the skull was exposed to reveal bregma. Guided by the atlas of the mouse brain (Paxinos & Franklin, 2001), a hole (1mm in diameter) was drilled, with a trepanning drill burr, directly above the brain area of interest (see Fig 2.4). The coordinates of the two brain regions studied in this project are as follows (from bregma; in mm):

• The M2 region of the PFC:	AP +2.10, ML +1.0, DV -2.0
• The dorsal Striatum:	AP +1.10, ML +1.5, DV -3.3

The dura was broken with a needle. Two self-tapping screws (Bioanalytical Systems) were attached to the skull to anchor the probe with cyanoacrylate gel (RS Components, Ltd) after probe insertion. The inserted microdialysis probe was primed with the modified Ringer's solution, and both of its in- and outlet ends were sealed with the gel to minimize the amount of air entering the probe, which could cause air locks during perfusion later in the experiment (see Fig 2.4).



Vital signs were monitored throughout the surgical process:

- Respiration was ensured at a rate of 100 ~ 190 times/min.
- Hypothermia was prevented by placing mice on a plastic bottle containing warm water.
- The colour of the mouse skin was monitored carefully to ensure cyanosis did not occur.

Fig 2.4 Photograph showing a mouse during the surgery. A microdialysis probe was inserted into the M2 region of the mouse prefrontal cortex under anaesthesia.

- **Recovery from anaesthesia**

After the surgery, mice were returned to their individual home cages for overnight recovery. Food and water were provided and standard lab conditions were applied (i.e. 12:12 h light / dark cycle (light on at 08:00 h), with temperature and humidity controlled at 20 ± 2°C and 45 ± 5%, respectively). Experiments were carried out the following day.

2.3.4.3. Collection of dialysates

Fig 2.5 Photograph showing the set-up in microdialysis sampling.

The day after surgery, microdialysis sampling was carried out, starting at 09:00 h. Mice were transferred to the experimental laboratory while still in their home cages. The inlet of the probe was connected, through FEP tubing (i.d.: 0.12 mm, o.d.: 0.68 mm; Royem Scientific Ltd.), to a 1 ml disposable syringe (BD Plastipak™), which was mounted in a perfusion pump (model: ESA/582; ESA Inc. Dorton, England), running at a flow rate of 1.5 μl / min. The outlet of the dialysis probe was

connected, through FEP tubing, to a 1.5 ml Eppendorf tubes (TreffLab, Switzerland), in which the samples were collected at 20-min intervals. Above the cage, a liquid swivel was held in a clamp to guide the inlet and outlet tubing of the probe, and to enable the mice to move freely during the experiment without entangling the connective tubing (see Fig 2.5).

Before starting the experiment, probes were allowed to equilibrate for at least 90 min. Then a minimum of three successive samples ('basal' efflux) were collected to establish the stable resting extracellular concentration of the neurotransmitter. Once steady-state basal samples were obtained, the test drugs were administered. Two distinct routes of administration were used in this project----local infusion ('retrodialysis') or systemic intraperitoneal (i.p.) injection.

- *Local administration* (e.g. K^+ salts or *d*-AMP; see Chapter 3 and 5, respectively): When the test compounds were given locally in the brain area of interest, the probe was first perfused with the modified Ringer's solution to establish the basal efflux level. Once steady efflux was reached, the treatment was given by changing the perfusion medium to a modified Ringer's solution in which the test compound was freshly dissolved. If necessary, withdrawal of the drug challenge was accomplished by reintroducing the pure modified Ringer's solution as the perfusion medium.

- *Systemic administration* (e.g. intraperitoneal injection of an NK1R antagonist or *d*-AMP; See Chapter 6): When drugs were given by i.p. injections, the modified Ringer's solution was continuously perfused down the probe throughout the experiment. Drug was injected at specific time points as stated in the protocols detailed in later chapters.

After collecting dialysate samples (30 µl / 20 min), analysis of the neurotransmitter content was performed immediately, using HPLC coupled to ECD.

2.3.5. Measurement of dialysate contents using HPLC coupled with ECD

Microdialysate samples usually comprise of low neurotransmitter concentrations in small volumes, and are limited by the compromise between maximal probe recovery and optimal spatial / time resolution. Thus, detection and quantification of the neurotransmitter content of the dialysate requires a highly sensitive technique. Among the different analytic techniques available nowadays (e.g. mass spectrometry and capillary electrophoresis, etc.), high-performance liquid chromatography with electrochemical detection (HPLC-ECD) is perhaps the most widely used analytical tool for separation and identification of biogenic amines, due to it sensitivity and specificity. This technique also offers a great advantage over other assays (e.g. mass spectrometry), as drugs can be administered in the knowledge of stable baseline values.

The HPLC-ECD assay has been used in this project to study changes in noradrenaline and / or dopamine efflux in different brain areas in NK1R+/+ and NK1R-/- mice, following drug administration.

2.3.5.1. The HPLC system

- **History**

Laboratory chromatography dates back to 1903, when the Russian chemist Mikhail Tswett first lectured on the separation of the pigments in green leaves on a chalk column. He then came up with the term 'chromatography' based on the coloured zones he observed on his chalk column (see Mefford, 1985).

In 1952, Archer John Porter Martin and Richard Laurence Millington Synge were awarded the Chemistry Nobel Prize for their invention of partition chromatography. Since then, the technology has advanced rapidly.

Researchers found that the principles underlying Tswett's chromatography could be applied in many different ways, giving rise to the different varieties of chromatography. Liquid chromatography separates ions or molecules that are dissolved in a solvent. In the 1970s, there

was a tremendous expansion in the use of liquid chromatography. This was due to its technical advances, such as the ability of the chromatography to work under higher pressures and flow rates, as well as the use of thinner columns and a variety of mobile phases.

Nowadays, high-performance liquid chromatography (HPLC), a form of liquid chromatography, has become increasingly popular in measuring compounds of interest obtained from microdialysis. Compared with methods based on, for example colorimetry, HPLC is much more rapid, sensitive and selective (Gorbunov & Esposito, 1991).

- **Principle of HPLC**

Chromatography covers a wide variety of separation techniques based on the partitioning of a solute between a mobile phase (which can be gas or liquid) and a stationary phase (which is normally a liquid or solid). There are two types of chromatographic packing which are most compatible with these requirements: ion-exchange and reverse-phase ion-pair chromatography (Riley *et al*, 1994).

- **Ion-exchange chromatography**

This involves the use of a charged stationary phase, usually an ion exchange resin that carries charged functional groups which interact with oppositely charged groups of the compound to be retained. In this way, charged compounds (e.g. amino acids and proteins) can be separated. This type of chromatography is commonly used to purify proteins using fast protein liquid chromatography, and is not discussed in detail in this thesis.

- **Reverse-phase ion-pair chromatography**

This type of chromatography was used in this project. In contrast to ion-exchange chromatography, this technique is employed to separate catecholamines and 5-HT. It consists of a non-polar stationary phase and a polar mobile phase. The analyte is forced through a column (stationary phase; normally silica-based) by an aqueous organic eluent containing a detergent (mobile phase) at high pressure. Separation is achieved through different migration rates of solutes. The more polar the substance, the faster it elutes from the non-polar stationary phase. By changing the pH of the mobile phase, the polarity of the analyte can be adjusted. This can modify

the time of different solutes forming an ion-pair with the detergent (i.e. the retention time). Individual substances are identified by their retention time, which is the characteristic time it takes for a particular molecule to pass through the column and enter the electrochemical cell under set conditions.

As mentioned above, the mobile phase plays a crucial role in determining the retention time of solutes in the dialysate. The mobile phase should be made up in the optimal composition to ensure separation of all the solutes and an acceptable range of retention times in order to achieve the required sensitivity for microdialylsis studies.

By modifying the mobile phase, the retention time of the solutes can be changed (Sharp & Zetterstrom, 1992):

Modification of mobile phase	**Effect on the retention time**
Increase in pH (only if p*K* differs from 7)	Decrease
Increase in concentration of detergents (e.g. OSA)	Increase
Increase in concentration of organic solvent	Decrease

In this project, two types of mobile phase (i.e. buffer) were used to detect noradrenaline and dopamine, respectively. These two buffers were made of the same components, but in different compositions:

Components and pH		**NA buffer**	**DA buffer**
NaH_2PO_4	(99%, Fischer)	100 mM	83 mM
Octanesulfonic acid (OSA)	(98%, Sigma)	2 mM	0.23 mM
EDTA	(99.5%, AnalarR)	0.67 mM	0.84 mM
Methanol	(99.8%, Prolabo)	12%	17%
pH, adjusted with orthophosphoric acid	(85%, BDH)	3.75	4.0

The buffer was degassed and filtered, before putting onto the system, with FTK membrane filters (removal rating: 0.1 μm; Whatman International Ltd). It was recycled for 3 ~ 4 months.

- **Characteristics of the HPLC system**

The HPLC system used in this project consists of the following (also see Fig 2.6):

Equipment	Function
A Shimadzu LC 6A isocratic dual piston pump (ESA 582)	Delivers the mobile phase to the system at a constant rate of 1.0 ml/min.
An in-line pulse damper (ESA)	Used to reduce pump noise.
A 50 µl stainless steel injection loop (Anachem) fitted with a Rheodyne 7125 injection port	This is where the dialysates are injected into the system.
An aquapore guard column: 7 µM particular size; 4.6 x 30 mm (Brownlee, Perkin Elmer)	To protect the main column.
A Hypersil octadecylsilane (ODS) column: 5 µM; 4.6 x 250 mm (Thermo Hypersil Ltd., UK)	This is where solutes are separated and measured. The stationary phase consists of silica derivatized with octadecyl groups. The silica-based stationary phase forms a hydrophobic bond with the detergent passing through it. Polar amines can form an ion-pair with the detergent. The rate at which solutes go past the column depends on: • the surface area of the column (related to diameter of the spherical packing material) • the type of hydrophobic layer • the amount of residual silanol groups on silica surface, which can give unwanted interactions with solutes

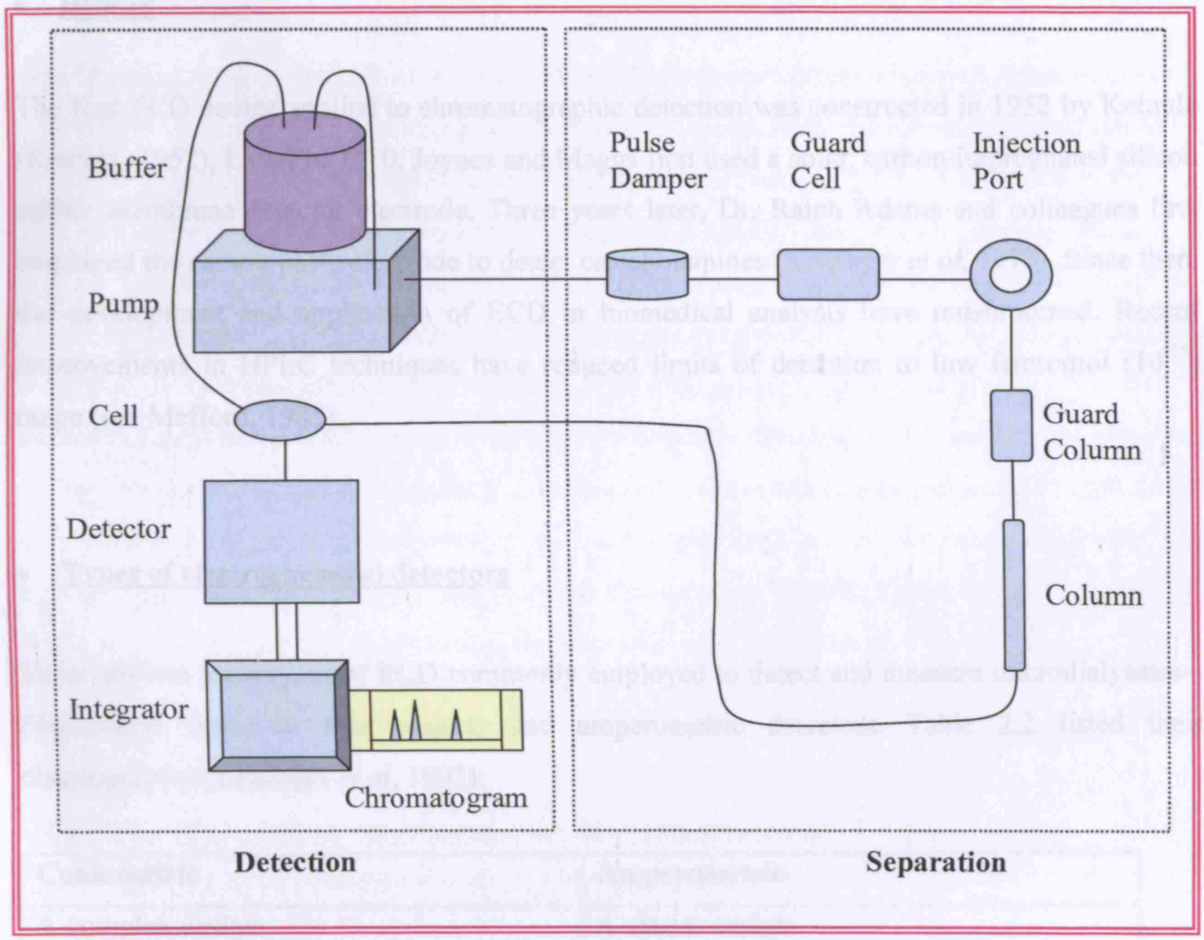


Fig 2.6 Schematic diagram of the HPLC-ECD used in this project.

2.3.5.2. The ECD system

The choice of detectors used in the HPLC analysis of microdialysis samples depends on the analytes in question and sensitivity of the analytical system. Many devices are presently employed for neurochemical and / or pharmacokinetic studies that use microdialysis sampling, such as fluorescence (Hadwiger *et al*, 1994), mass spectrometry (Kissinger *et al*, 1991) and electrochemistry (amperometry). This project used electrochemical detection (ECD), which is well established for amino acids, biogenic amines (NA and DA), 5-HT and their metabolites (Marsden *et al*, 1990; Cheng & Kuo, 1995).

- **History**

The first ECD device applied to chromatographic detection was constructed in 1952 by Kemula (Kemula, 1952). Later, in 1970, Joynes and Maggs first used a solid, carbon-impregnated silicon rubber membrane detector electrode. Three years later, Dr. Ralph Adams and colleagues first employed the carbon paste electrode to detect catecholamines (Kissinger *et al*, 1973). Since then, the development and application of ECD in biomedical analysis have mushroomed. Recent improvements in HPLC techniques have reduced limits of detection to low femtomol (10^{-15}) range (see Mefford, 1985).

- **Types of electrochemical detectors**

There are two main types of ECD commonly employed to detect and measure microdialysates--coulometric (used in this project) and amperometric detectors. Table 2.2 listed their characteristics (Kissinger *et al*, 1992):

Coulometric	**Amperometric**
A complex design: Consists of a large active electrode surface area to be exposed to the solution, which leads to a higher background noise.	A simple design: Consists of an electrode, which is part of a channel wall, formed by a fluorocarbon gasket (typically 50-80 μm) between two inert plastic blocks. The cell volume can be reduced to 0.15-0.25 μl by using a thinner gasket.
Attempt to achieve 100% conversion of electroactive species of interest.	Attempt to convert as little as possible (1 ~ 5%) of the electroactive species in solution.
Large dead volume (> 2 μl).	Small dead volume (< 1 μl).

Table 2.2 Comparison of coulometric and amperometric detectors.

- **Principle of ECD**

After passing through the HPLC column, solutes reach the electrochemical cell, where detection of analytes occurs. The electrochemical detection is based on a reduction-oxidation reaction, which takes place inside the cell. The cell can be made of various types of electrode materials, including carbon, mercury and platinum (see Mefford, 1985). In this project, the cell consisted of two glassy carbon electrodes (useful for oxidizable and easily reducible species) placed in series. At the first electrode, which conditions the mobile phase containing solutes, a negative potential is applied by donating an electron (reduction). The released electrons are accepted by the second electrode (oxidation), which is set at a positive potential. Therefore, the transfer of electrons during the oxidation reaction generates a current, which is measured by the detector. According to equation 2.1, the current produced (i_L) is proportional to the difference between the concentration of solute entering and leaving the detector, the flow rate and the amount of electrons exchanged in the conversion (Riggin *et al.*, 1976; Swartzfager, 1976):

$$i_L = K^{1-b} n F (C_e - C_l) V_f^{a} \qquad \text{Equation 2.1}$$

where K = a constant incorporating all the various factors except the flow rate

n = the number of electrons per molecule of reacting species exchanged in the conversion

F = the Faraday constant

C_e and C_l = the concentration of solute entering and leaving the detector, respectively

V_f = the flow rate

a and b are dependent on electrode geometry, flow patterns and the efficiency of the electrochemical reaction

Under coulometric conditions, C_l is zero, a and b are unity, and the flow rate is constant. Hence the current response is directly proportional to the concentration of electroactive solute in the dialysate that reaches the detector (see Equation 2.2.), and this is expressed as a peak on the chromatogram by the integrator:

$$i_L = B C_b \qquad \text{Equation 2.2}$$

where B = a constant incorporating all the various parameters, including flow rate

C = the concentration of electroactive solute present

Detector potential (*E*)

Choosing the appropriate detector potential is very important because it can alter the rate of the oxidation and reduction reactions at the electrodes, and can therefore vary the current produced. The applied potential should be sufficiently low to minimize background current and the noise caused by solvent turbulence at the electrode, yet sufficiently high to oxidize or reduce as much solute as possible.

For the set-up used in this project, the conditioning electrode was set at -280 mV, according to previous studies done by colleagues. On the other hand, current / potential calibration curves (voltammograms) for NA and DA was constructed, in order to evaluate an optimal oxidizing potential for the measuring electrode. Here, 50 μl of 60 fmol NA standard solution (100 fmol solution in the case of DA) was injected into the HPLC-ECD system, which was set at a range of potentials from -50 to +280 mV. In the voltammograms obtained (see Fig 2.7), a plateau was observed from +140 to +280 mV, whereas from +140 mV downwards NA and DA peak height decreased sharply and linearly as voltage became smaller. As a result, +140 mV was chosen as the optimal potential for the measuring electrode when detecting NA or DA contents.

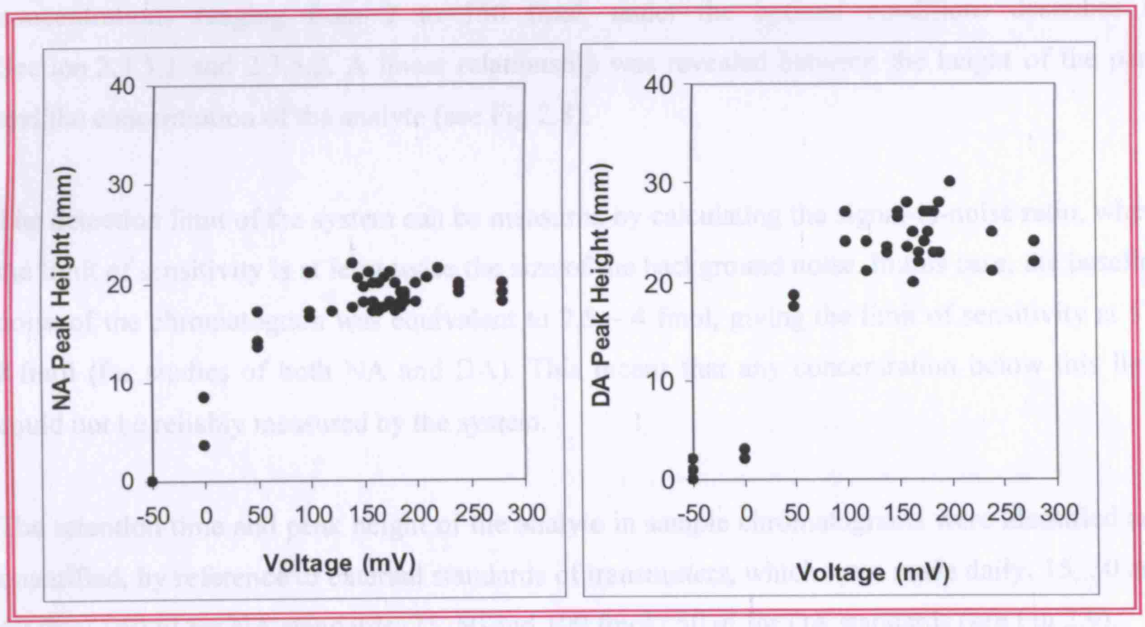


Fig 2.7 Voltammograms demonstrating the optimal oxidation potential for NA (left) and DA.

- **Characteristics of the ECD system**

The ECD system used in this project consisted of the following equipment:

Equipment	Function
A guard cell (5020, ESA)	It is set at +350 mV and used to condition the mobile phase.
An electrochemical cell (Coulochem 5014, ESA), consisting of two electrodes in series.	Detection and measurement: • conditioning electrode: set at -280 mV for reduction • measuring electrode: set at +140 mV for oxidation
An electrochemical detector (Coulochem II 5100A, ESA)	Displaying currents and voltages
An integrator (Chromiet, Spectra-Physics)	Data capture

2.3.5.3. Calibration of the HPLC-ECD system

To ensure accurate measurement of analytes, it was important to calibrate the HPLC-ECD system in a broad range of concentrations of the analyte and to determine the detection limit of the assay, before starting the project. This was performed by injecting 50 µl of NA or DA at concentrations ranging from 2 to 150 fmol, under the optimal conditions describes in Section 2.3.5.1 and 2.3.5.2. A linear relationship was revealed between the height of the peak and the concentration of the analyte (see Fig 2.8).

The detection limit of the system can be measured by calculating the signal-to-noise ratio, where the limit of sensitivity is at least twice the size of the background noise. In this case, the baseline noise of the chromatogram was equivalent to 2.5 ~ 4 fmol, giving the limit of sensitivity at 5 ~ 8 fmol (for studies of both NA and DA). This means that any concentration below this limit could not be reliably measured by the system.

The retention time and peak height of the analyte in sample chromatograms were identified and quantified, by reference to external standards of transmitters, which were made daily: 15, 30 and 60 fmol / 50 µl for NA standards; 25, 50 and 100 fmol / 50 µl for DA standards (see Fig 2.9).

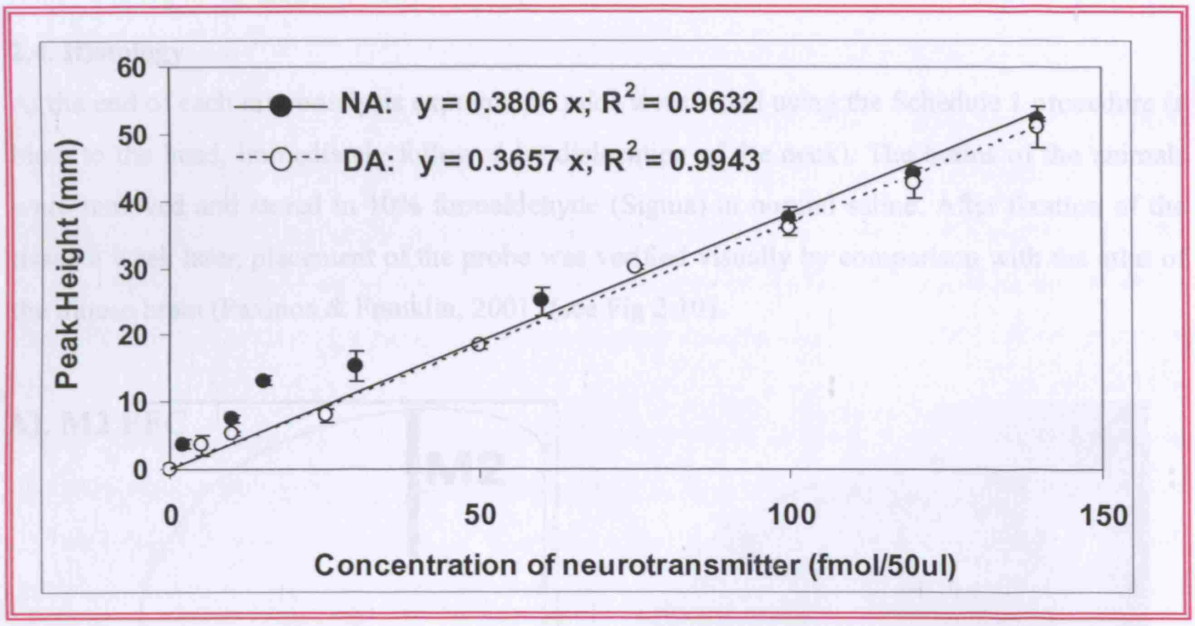


Fig 2.8 Calibration curves for detection of NA and DA: constructed by applying different concentrations of the catecholamines (2 ~ 150 fmol / 50 μl).

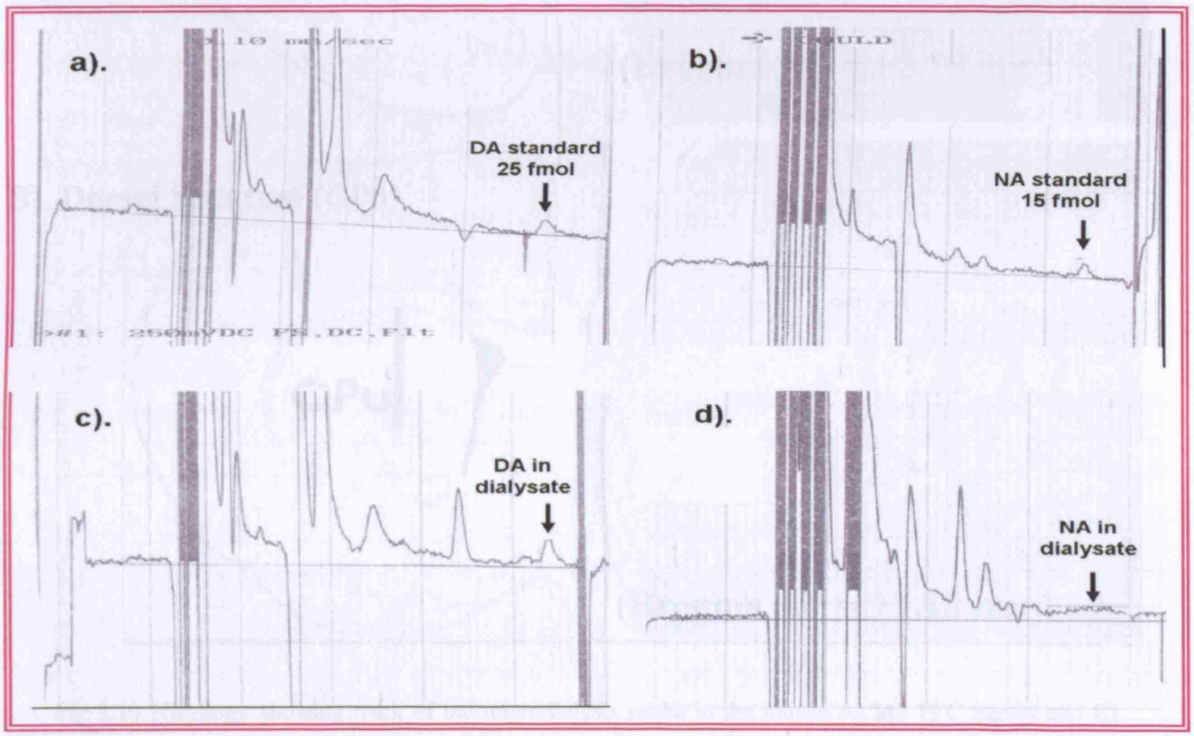


Fig 2.9 Traces of chromatograms showing standard solutions of: a) DA and b) NA, as well as concentrations of: c) DA and d) NA in the dialysate.

2.4. Histology

At the end of each microdialysis experiment, mice were killed using the Schedule 1 procedure (a blow to the head, immediately followed by dislocation of the neck). The brains of the animals were removed and stored in 10% formaldehyde (Sigma) in normal saline. After fixation of the tissue a week later, placement of the probe was verified visually by comparison with the atlas of the mouse brain (Paxinos & Franklin, 2001) (see Fig 2.10).

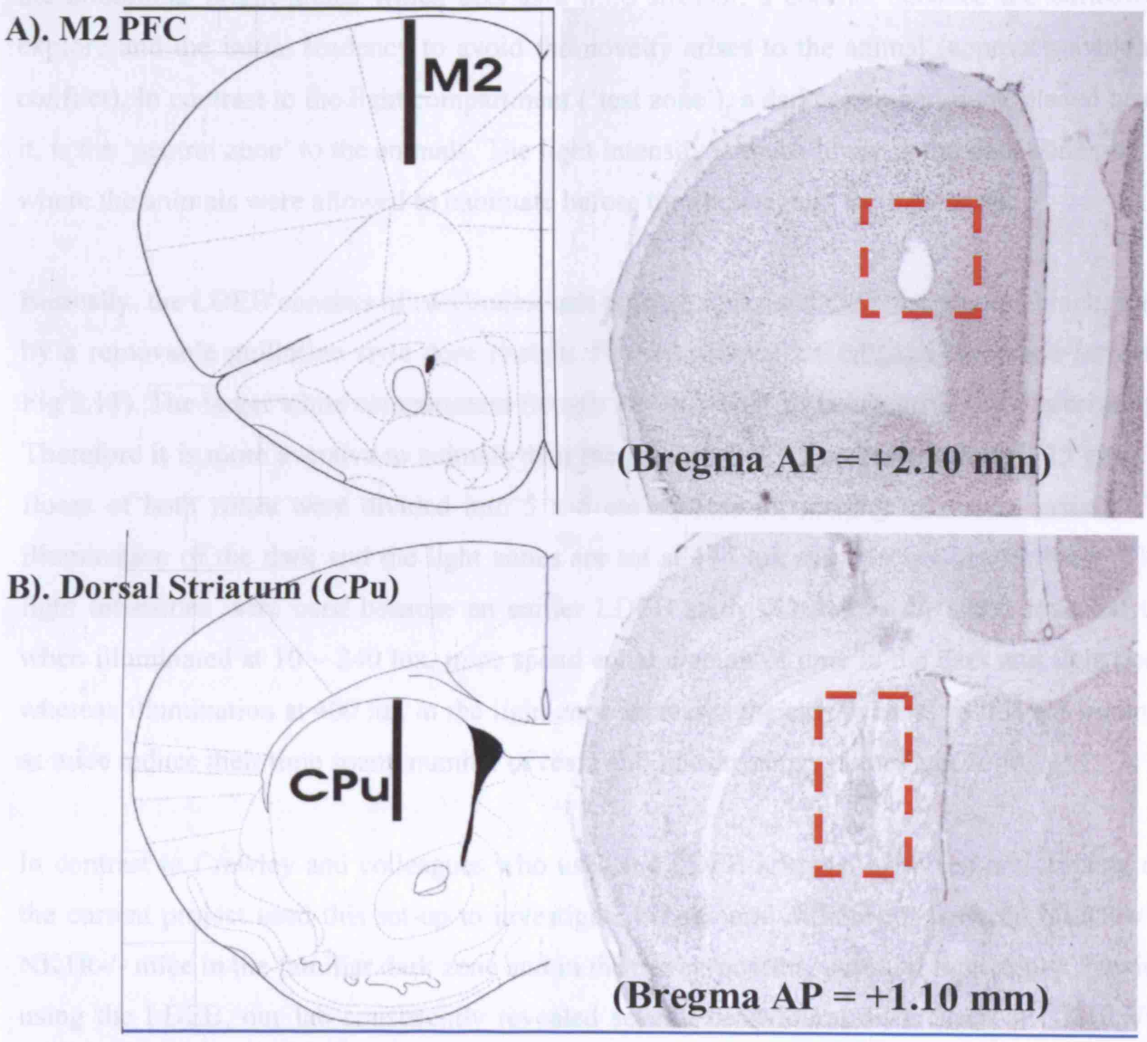


Fig 2.10 Histology showing track of the microdialysis probe in the mouse A) M2 PFC region and B) dorsal striatum. The schematic graphs (left) are obtained from Paxinos & Franklin (2001). Not drawn to scale. Line indicates the dialysis window. The dotted square (right) indicates probe track. Probe placement was checked randomly. In all cases, the probe was correctly inserted in the target brain region.

2.5. Behaviour

2.5.1. Light / Dark Exploration Box----apparatus

The light / dark exploration box (LDEB) was first described by Crawley & Goodwin in 1980. This paradigm is based on an innate aversion of rodents to the brightly lit environment and on the spontaneous exploratory behaviour of the animals (see Hascoet *et al.*, 2001). Once placed in the unfamiliar bright arena, which acts as a mild stressor, a conflict between the curiosity to explore and the initial tendency to avoid the novelty arises to the animal (approach-avoidance conflict). In contrast to the light compartment ('test zone'), a darker compartment, placed next to it, is the 'neutral zone' to the animals. The light intensity is much lower in the dark zone and it is where the animals were allowed to habituate before the challenge in the light zone.

Basically, the LDEB consists of two boxes, one painted white and the other painted black, joined by a removable guillotine style door (height 10.5 cm; width 7.5 cm; see the green arrows in Fig 2.11). The larger white compartment (length 30 cm; width 20 cm; depth 25 cm) is brightly-lit. Therefore it is more aversive to animals than the smaller dark compartment (length 15 cm). The floors of both zones were divided into 5 x 5 cm squares for scoring locomotor activity. The illumination of the dark and the light zones are set at 174 lux and 346 lux, respectively. These light intensities were used because an earlier LDEB study (Costall *et al*, 1989) revealed that, when illuminated at 10 ~ 240 lux, mice spend equal amount of time in the dark and light zones, whereas illumination at 400 lux in the light zone increases the aversiveness of this environment, as mice reduce their time spent, number of rears and line crossings in the light zone.

In contrast to Crawley and colleagues who used the LDEB as a test to screen anti-anxiety drugs, the current project used this set-up to investigate behavioural differences between NK1R+/+ and NK1R-/- mice in the familiar dark zone and in the novel (possibly stressful light zone). Previously, using the LDEB, our lab consistently revealed several behavioural differences in NK1R+/+ and NK1R-/- mice (Herpfer *et al*, 2005; Fisher *et al*, 2007). In particular, NK1R-/- mice display increased locomotor activity, rearing and time spent in the light zone, but they exhibit decreased risk assessment behaviours (flat-back approach and stretch-attend posture). The LDEB test enables

a wide range of behaviour to be monitored. Therefore, this paradigm acts as a broad brush that pulls out various genotype differences, indicating what behaviours to explore in more detail in future work.

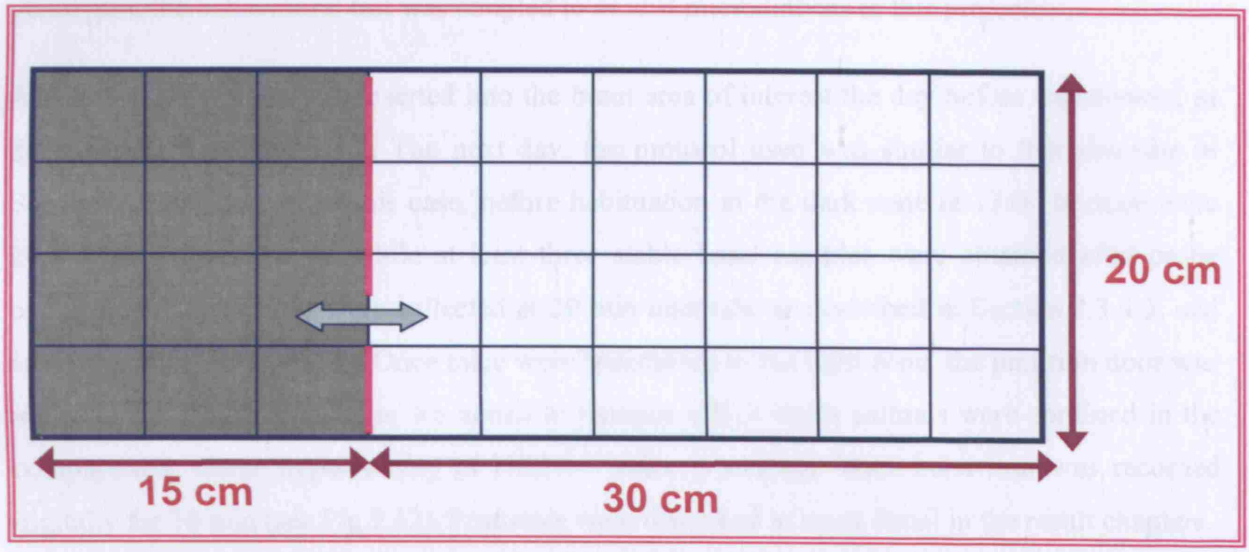


Fig. 2.11 Schematic of the light / dark exploration box (LDEB) used in this project.

2.5.2. Behaviour test in the LDEB

Animals were run in pairs, using two LDEB placed side by side. All behavioural tests were performed in the LDEB, starting at 13:00 h. Mice were first allowed to habituate in the dark zone for 1.5 h. During the habituation, intraperitoneal injection(s) of drug(s) was given (at 13:30 h and 14:00 h if two drugs were administered; at 14:00 h if one drug was administered). Mice were returned to the dark zone after the injection(s). At 14:30 h, mice were transferred to the light zone with their heads facing away from the guillotine style door. In the meantime, the door was removed to allow mice to commute freely between the two compartments. The behaviour of the animals was recorded with a Sony Handycam Vision video recorder for 30 min, and various behaviours (see Section 5.1) were scored 'blind'. Protocols are described in more detail in the Results chapters.

2.5.3. *In vivo* microdialysis in the LDEB

Previously in this lab, colleagues have revealed that NK1R+/+ and NK1R-/- mice behaved differently in a number of respects in the LDEB (Herpfer *et al*, 2005; Fisher *et al*, 2006). In order to find out the neurochemical explanation for the behavioural differences between the two genotypes, the behavioural test was coupled to *in vivo* microdialysis in this project.

A microdialysis probe was inserted into the brain area of interest the day before experiment, as described in Section 2.3.4.2. The next day, the protocol used was similar to that describe in Section 2.5.2. However in this case, before habituation in the dark zone at 13:00 h, mice were kept in their home cages, while at least three stable basal samples were obtained after probe equilibration. Dialysates were collected at 20-min intervals, as described in Section 2.3.4.3, and analyzed using HPLC-ECD. Once mice were transferred to the light zone, the partition door was *not* removed this time because we aimed to monitor efflux while animals were confined in the compartment where hyperactivity of NK1R-/- mice is evident. Mice behaviour was recorded digitally for 30 min (see Fig 2.12). Protocols were described in more detail in the result chapters.

Fig 2.12 Photograph showing the set-up used in the *in vivo* microdialysis studies of mice in the LDEB. Liquid swivels were attached to the wall of the light zone to guide the connective tubings as mice moved.

2.6. Statistics

2.6.1. Analysis for microdialysis data

SPSS PC$^+$ for Windows was used to analyze the data by repeated-measures analysis of variance (ANOVA). Typically, a 3-way (e.g.'time'*'genotype'*'drug'), 2-way or 1 way (where appropriate) ANOVA was performed.

The NA or DA content is expressed as fmol / 20 min without correction for probe recovery. Routinely, statistical analysis was carried out on both the raw data and the net change in efflux

caused by treatments. The net changes were calculated by subtracting the mean efflux in the three basal samples (collected immediately before the treatment) from all samples (including the basals). This normalizes treatment groups with respect to basal efflux, thus facilitating the comparison of the effects of drugs (see Dalley *et al*, 1996).

For the analysis with repeated measures ANOVA, 'Time' was considered as a 'within-subjects' factor. 'Genotype' and 'Drug' were considered as 'between-subject' factors when comparing between genotypes and effects of different drugs, respectively. Further, in the case of the drug-induced response *vs.* basals, or comparing two treatments within the same animal, the data were separated into 'bins' of three or more consecutive samples. In this case, the 'within-subject' factors would be 'bins' of time points.

The Greenhouse-Geisser 'ε' correction was applied to compensate for any violation of the Mauchley's test of sphericity. $P \leq 0.05$ was the criterion for statistical significance for all tests.

2.6.2. Analysis for behavioural data

Behavioural data were first analyzed using multivariate analysis followed by multifactorial ANOVA, where 'Genotype' and 'Drug' were considered as main factors. Once significance was reached on a main effect of the factors or their interaction, Post-Hoc multiple, using the LSD test, were performed to explore any difference between individual groups. Levene's test of homogeneity of variance was performed routinely. If this is significant, non-parametric analysis was performed using the Mann-Whitney test.

In the behavioural experiment, locomotor activity could be confounding the drug effects on other behaviours and hence it was regarded as a 'covariate'. Therefore, analysis of covariance (ANCOVA) was performed to determine whether any differences in the behavioural measures could be masked or explained by differences in locomotor activity. $P \leq 0.05$ was set as the criterion for significance.

2.7. Drugs and reagents

d-AMP and MPH were purchased from Sigma-Aldrich (Poole, UK). RP 67580, L 733060 and RX 821002: Tocris (Bristol, UK). HPLC buffer reagents: either AnalaR or HPLC grade (BDH).

Chapter 3. The effects of $[K^+]_o^{\uparrow}$-induced depolarization and $[Ca^{2+}]_o$ depletion on noradrenaline efflux in the prefrontal cortex of NK1R+/+ and NK1R-/- mice

3.1. Introduction

Transmitters can be released from nerve terminals to extracelluar space by the following mechanisms:

- Impulse-evoked release ('exocytosis'): caused by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as a result of neuronal impulses (see below).
- Impulse-independent release: does not rely on neuronal activation. Release occurs even in the absence of $[Ca^{2+}]_i$ (e.g. release induced by *d*-AMP; see Chapter 4).
- Leakage: transmitters might leak into extracellular space from terminals due to, for example, tissue damage.

When setting up microdialysis studies, it is important to first confirm that the extracellular neurotransmitters being monitored are of a neuronal origin and are released impulse-dependently. This can be tested by monitoring changes in transmitter efflux in response to: (i) depolarization caused by increased extracellular concentration of K^+ ($[K^+]_o^{\uparrow}$), which causes presynaptic activation; (ii) depletion of Ca^{2+} ions (which are needed for stimulus-secretion coupling) in the perfusion medium.

$[K^+]_o^{\uparrow}$–evoked depolarization can be carried out by increasing extracellular K^+ concentration to 80 mM. In consequence, these ions enter the cell down their concentration gradient, which then induces depolarization of the plasma membrane. This, in turn, leads to the opening of the voltage-gated Na^+ channels situated on the cell membrane, and the influx of Na^+ ions down their concentration gradient, which causes further depolarization of the membrane (Den Hertog *et al*, 1976). In this way, challenges with high K^+ concentrations mimic neuronal stimulations.

The arrival of a propagated nerve impulse at neuronal terminals, or raising $[K^+]_o$, mobilizes Ca^{2+} from intracellular stores (e.g. the endoplasmic reticulum) and / or opens voltage-gated Ca^{2+}-channels on the plasma membrane, allowing influx of Ca^{2+} ions into the presynaptic nerve terminals. This causes a phasic increase in free $[Ca^{2+}]_i$ (from 75~100 nM to 0.1~1 mM) in the 'active zone' (the part of the nerve terminal where release occurs). Subsequently,

phosphorylation / dephosphorylation of synapsins (synaptic vesicle-associated protein) mediated by protein kinases (e.g. Ca^{2+}/calmodulin protein kinase II) occurs. This then moves vesicles from a storage pool to a release pool for exocytosis (Cuchillo-Ibanez *et al*, 2002; Augustine *et al*, 2003; Kuromi & Kidokoro, 2005). An increase in $[Ca^{2+}]_i$ is essential for various stages of the docking / fusion / extrusion cascade, and removal of Ca^{2+} ions from the perfusion medium can blunt impulse-dependent release of transmitters.

Previous studies have shown that the spontaneous cortical efflux of NA in rats rests on an impulse-evoked Ca^{2+}-dependent mechanism (L'Heureux *et al*, 1986; van Veldhuizen *et al*, 1990; Dalley & Stanford, 1995; Hughes & Stanford, 1998). Here I confirmed whether this is also true in mice and investigated whether the depolarization-induced NA efflux in the PFC differs in NK1R+/+ and NK1R-/-mice. Hence, the current study was performed to compare the effects of 80 mM $[K^+]_o$ challenge and / or removal of Ca^{2+} from the perfusion medium on NA release in the PFC of freely-moving NK1R+/+ and NK1R-/- mice, using *in vivo* microdialysis.

3.2. Aim

***In vivo* microdialysis was used to test the effects of local infusion of the following solutions into the PFC in freely-moving NK1R+/+ and NK1R-/- mice:**

- **Modified Ringer's solution containing an elevated $[K^+]_o$ (80 mM)**
- **Modified Ringer's solution containing 80 mM $[K^+]_o$ in which Ca^{2+} was replaced by Mg^{2+} ('Ca^{2+}-free')**

Rationale of experiments

In order to verify the impulse-dependency of NA release in NK1R+/+ and NK1R-/- mice, a series of experiments was performed:

3.2.1. **Double-pulse experiments** ('***K***$^+$ (Pulse 1) / ***Ca***$^{2+}$***-free*** (Pulse 2)' or '***K***$^+$ (Pulse 1) / ***K***$^+$ (Pulse 2)') was carried out in both genotypes. This experimental design was used because: (i) application of two K^+ pulses enabled us to test whether release of NA was maintained during consecutive stimulations in NK1R+/+ and NK1R-/- mice; (ii) when testing the effects of Ca^{2+} depletion, Pulse 1 K^+ would act as a control for Pulse 2 *Ca^{2+}-free*, thus each animal could act as its own control.

3.2.2. Since the effects of Ca^{2+} depletion in NK1R+/+ mice were not apparent after the above experiment, the double-pulse experiments were repeated, in another batch of wild-types, with the inclusion of the Ca^{2+} chelator, **EGTA**, in the *Ca^{2+}-free* pulse. This was to ensure a maximum Ca^{2+}-depleted environment, as there might be residual extracellular Ca^{2+} ions which diffuse from other sites.

3.2.3. Since depletion of Ca^{2+} still did not exhibit an apparent effect after using the aforementioned protocols, a **single pulse** (either K^+ or *Ca^{2+}-free+EGTA* pulse) was applied, in a new batch of wild-types. This protocol was used in order to test whether the Ca^{2+}-dependency in NK1R+/+ mice was masked by their lack of ability to keep up the NA response to the 2nd K^+ pulse.

3.3. Protocol

3.3.1. Double-pulse experiments

Once stable basal NA efflux was obtained, NK1R+/+ and NK1R-/- mice were randomly assigned to one of the following treatments (see Fig 3.1):

--Two successive pulses of local infusion of modified Ringer's solution containing 80 mM $[K^+]_o$ (mM: KCl 80; NaCl 71 and $CaCl_2$ 1.3, pH 6.8).

--Local infusion of 80 mM $[K^+]_o$-containing Ringer's solution (Pulse 1 in Fig 3.1), followed by infusion of 80 mM $[K^+]_o$-containing Ringer's solution in which $[Ca^{2+}]_o$ was replaced by $[Mg^{2+}]_o$ (Pulse 2 in Fig 3.1: referred to as 'Ca^{2+}-free pulse' in this chapter; pH 6.8;)

The test pulses each lasted for 40 min and were separated by 80 min. The normal Ringer's solution (see Section 2.3.4.1 for recipe) was reinstated after each pulse in order to restore spontaneous NA efflux.

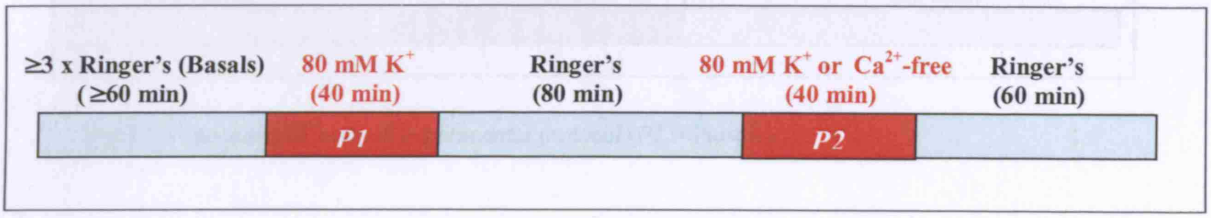


Fig 3.1 Time-matched scale of experimental protocol (*P1* = Pulse 1; *P2* = Pulse 2).

3.3.2. Double-pulse experiments (with EGTA)

In another batch of NK1R+/+ mice, the above protocol was repeated with the inclusion of the Ca^{2+} chelator, EGTA, in the *Ca^{2+}-free* pulse.

EGTA was dissolved in the following medium (mM): KCl 80, NaCl 71, $CaCl_2$ 8.2 x 10^{-3}, $MgCl_2$ 3.01, NaOH 2, HEPES 10 (pH 7.4). Calculation of concentrations of the reagents was performed using the website: *http://www.stanford.edu/~cpatton/webmaxc/webmaxcE.htm* (personal communication with Dr. Alasdair Gibb, UCL). This solution was well mixed at 37°C before infusion.

3.3.3. Single-pulse experiments (with EGTA)

In a new batch of NK1R+/+ mice, a single pulse of either 80 mM K^+ ***or*** Ca^{2+}-free (with EGTA) was given, followed by reinstatement of modified Ringer's solution (see Fig 3.2).

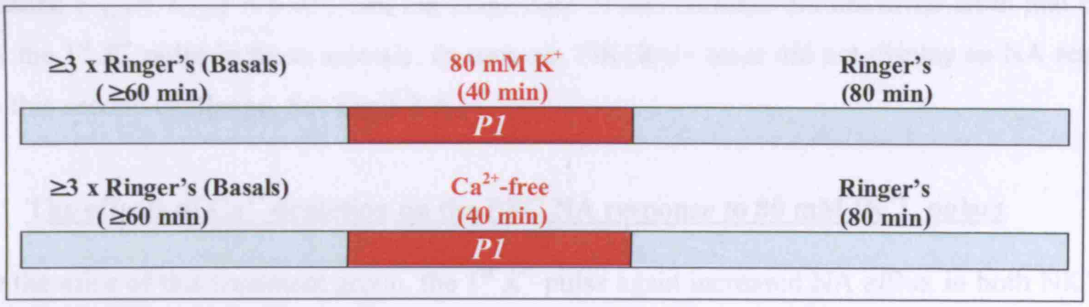


Fig 3.2 Time-matched scale of experimental protocol (*P1* = Pulse 1).

3.4. Results

3.4.1. Double-pulse experiments

Comparisons were performed between treatment groups, which all come from the same randomized study.

- **The effects of two successive 80 mM $[K^+]_o$ pulses**

In these treatment groups, the 1st K^+ pulse (P1) caused a prominent increase in cortical NA efflux in both NK1R+/+ (P1 K^+ *vs.* Basals: $F_{1, 10} = 31.1$, $P < 0.001$) and NK1R-/- mice (P1 K^+ *vs.* Basals: $F_{1, 9} = 10.1$, $P < 0.05$). See Fig 3.3 A.

However, when the 2nd K^+ pulse (P2) was given, the two genotypes responded differently. In NK1R-/- mice, NA efflux in the PFC was again elevated after the 2nd K^+ challenge (P2 K^+ *vs.* Basals: $F_{1, 9} = 7.7$, $P < 0.05$), and the magnitude of this increase did not differ from that caused by the 1st K^+ pulse in these animals. In contrast, NK1R+/+ mice did not display an NA response to this second challenge. See Fig 3.3 A.

- **The effects of Ca^{2+} depletion on the PFC NA response to 80 mM $[K^+]_o$ pulses**

In the mice of this treatment group, the 1st K^+ pulse again increased NA efflux in both NK1R+/+ (P1 K^+ *vs.* Basals: $F_{1, 9} = 10.1$, $P < 0.05$; see Fig 3.3 B) and NK1R-/- mice (P1 K^+ *vs.* Basals: $F_{1, 11} = 6.8$, $P < 0.05$; see Fig 3.3 C).

However, removal of Ca^{2+} from the 80 mM $[K^+]_o$-containing Ringer's solution at the 2nd pulse prevented the NA response in both NK1R+/+ (P2 Ca^{2+}-free *vs.* P1 K^+: $F_{1, 8} = 11.8$, $P < 0.01$, see Fig 3.3 B) and NK1R-/- mice (P2 Ca^{2+}-free *vs.* P1 K^+: $F_{1, 10} = 4.7$, $P < 0.05$; see Fig 3.3 C).

- **2nd 80 mM $[K^+]_o$ pulse *vs.* 2nd $[Ca^{2+}]_o$-free pulse**

When comparing the NA response to the 2nd K^+ pulse with that during the 2nd *Ca^{2+}-free* pulse, an effect of Ca^{2+} depletion was observed in NK1R-/- mice ($F_{1, 19} = 4.7$, $P < 0.05$, see Fig 3.3 C), but in not NK1R+/+ mice (see Fig 3.3 B).

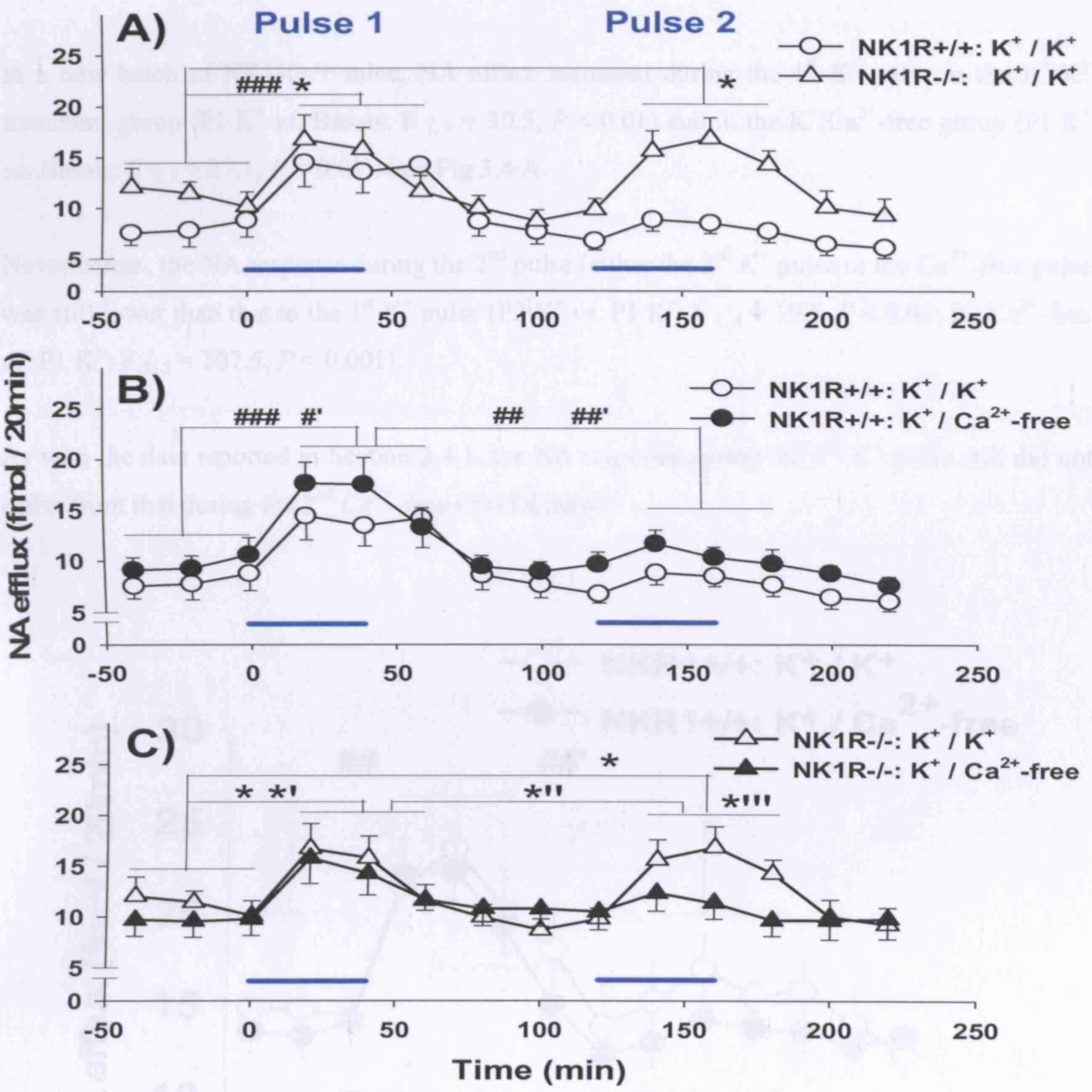


Fig 3.3 The PFC NA efflux in response to 80 mM K^+ stimulation(s) and / or Ca^{2+}-depletion. Fig A~C illustrate data from the same randomized study. Groups are illustrated in pairs, for clarity. **A)** The NA response to two successive 80 mM K^+ pulses in NK1R+/+ (P1 K^+ *vs.* Basals: ### $P < 0.001$) and NK1R-/- mice (P1 or P2 K^+ *vs.* Basals: * $P < 0.05$); **B)** The NA response in the NK1R+/+ mice that received K^+ / K^+ treatments (P1 K^+ *vs.* Basals: ### $P < 0.001$; P2 K^+ *vs.* P1 K^+: ## $P < 0.01$) and in the NK1R+/+ mice that received K^+ / Ca^{2+}-free treatments (P1 K^+ *vs.* Basals: #' $P < 0.05$; P2 Ca^{2+}-free *vs.* P1 K^+: ##' $P < 0.01$); **C)** The NA response in the NK1R-/- mice that received K^+ / K^+ treatments (P1 or P2 K^+ *vs.* Basals: * $P < 0.05$) and in the NK1R-/- mice that received K^+ / Ca^{2+}-free treatments (P1 K^+ *vs.* Basals: *' $P < 0.05$; P2 Ca^{2+}-free *vs.* P1 K^+: *'' $P < 0.05$; P2 Ca^{2+}-free *vs.* P2 K^+: *''' $P < 0.05$).

3.4.2. Double-pulse experiments (with EGTA)----in NK1R+/+ mice only

In a new batch of NK1R+/+ mice, NA efflux increased during the 1st K^+ pulse in the K^+/K^+ treatment group (P1 K^+ *vs.* Basals: $F_{1,5} = 30.5$, $P < 0.01$) and in the K^+/Ca^{2+}-free group (P1 K^+ *vs.* Basals: $F_{1,5} = 27.1$, $P < 0.01$). See Fig 3.4 A.

Nevertheless, the NA response during the 2nd pulse (either the 2nd K^+ pulse or the *Ca^{2+}-free* pulse) was still lower than that to the 1st K^+ pulse (P2 K^+ *vs.* P1 K^+: $F_{1,4} = 19.5$, $P < 0.05$; P2 Ca^{2+}-free *vs.* P1 K^+: $F_{1,5} = 107.5$, $P < 0.001$).

As with the data reported in Section 3.4.1, the NA response during the 2nd K^+ pulse still did not differ from that during the 2nd *Ca^{2+}-free+EGTA* pulse.

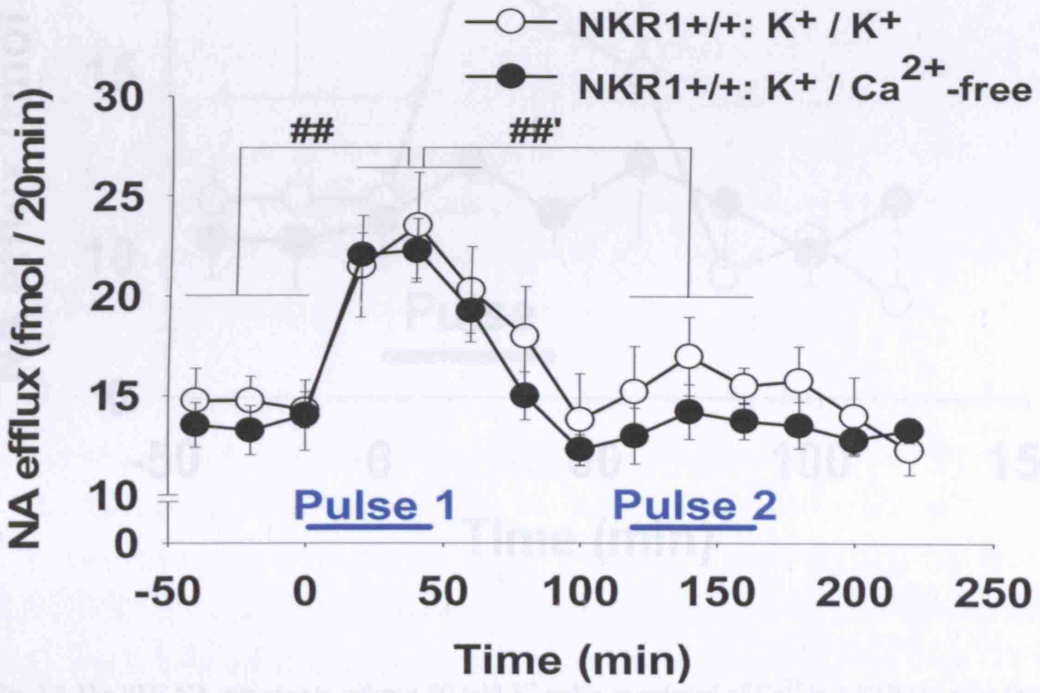


Fig 3.4 The PFC NA response to 80 mM K^+ and / or Ca^{2+}-free+EGTA pulses in a new batch of NK1R+/+ mice (N = 6 / group). For both K^+/K^+ and K^+/Ca^{2+}-free treatment groups, P1 K^+ *vs.* Basals: ## $P < 0.01$; P2 K^+ or P2 Ca^{2+}-free *vs.* P1 K^+: ##' $P < 0.01$).

3.4.3. Single-pulse experiments (with EGTA) ----in NK1R+/+ mice only

In another batch of NK1R+/+ mice, a single pulse of 80 mM $[K^+]_o$ augmented NA efflux by 60% (T20~60 *vs.* basals: $F_{1,2} = 71.1$, $P < 0.05$). This 80 mM $[K^+]_o$-induced NA response was abolished after removing Ca^{2+} from the perfusion medium, in which EGTA was added (T20~60: K^+ pulse *vs.* Ca^{2+}-free pulse: $F_{1,4} = 13.5$, $P < 0.01$). See Fig 3.5.

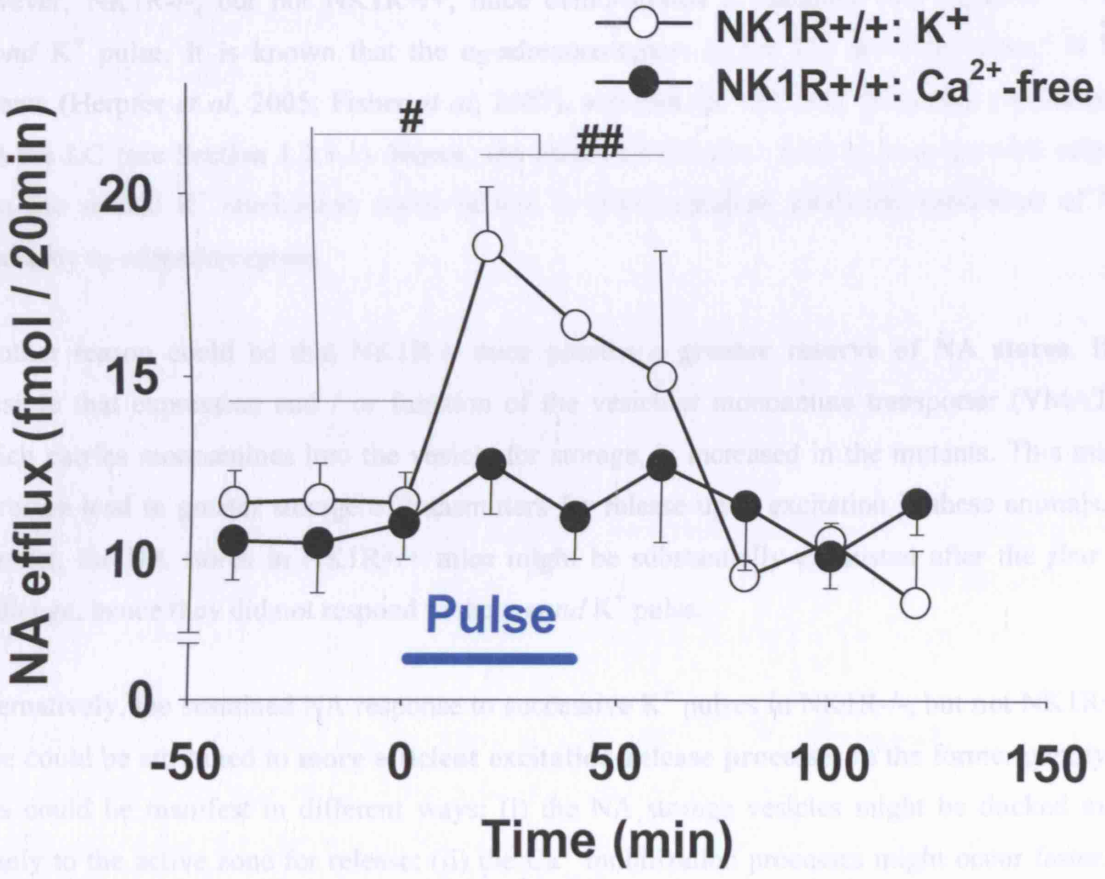


Fig 3.5 The PFC NA response to either a 80 mM K^+ pulse or removal of Ca^{2+} in NK1R+/+ mice (N = 3 / group). K^+ *vs.* Basals: # $P < 0.05$; K^+ *vs.* Ca^{2+}-free: ## $P < 0.01$.

3.5. Discussion

3.5.1. Double-pulse experiments

- **The effects of two successive high-K^+ pulses (i.e. K^+ / K^+)**

The results described in this chapter indicated that NA release in the PFC is impulse-dependent in both NK1R+/+ and NK1R-/- mice, as NA efflux was enhanced by 80 mM $[K^+]_o$ stimulation in both genotypes.

However, NK1R-/-, but not NK1R+/+, mice demonstrated a sustained NA response to the *second* K^+ pulse. It is known that the α_2-adrenoreceptors in the LC are desensitized in the mutants (Herpfer *et al*, 2005; Fisher *et al*, 2007), and that the PFC has reciprocal connectivity with the LC (see Section 1.2.1.1). Hence, the ability of NK1R-/- mice to keep up with release upon the second K^+ stimulation could be due to their **impaired feedback inhibition** of NA release by α_2-adrenoreceptors.

Another reason could be that NK1R-/- mice possess a **greater reserve of NA stores**. It is possible that expression and / or function of the vesicular monoamine transporter (VMAT2), which carries monoamines into the vesicle for storage, is increased in the mutants. This might therefore lead to greater storage of transmitters for release upon excitation in these animals. In contrast, the NA stores in NK1R+/+ mice might be substantially exhausted after the *first* K^+ challenge, hence they did not respond to the *second* K^+ pulse.

Alternatively, the sustained NA response to successive K^+ pulses in NK1R-/-, but not NK1R+/+ mice could be attributed to **more efficient excitation-release processes** in the former genotype. This could be manifest in different ways: (i) the NA storage vesicles might be docked more closely to the active zone for release; (ii) the Ca^{2+} mobilization processes might occur faster. In particular, SNAP-25, a presynaptic plasma membrane protein (Oyler *et al*, 1989; see Söllner *et al*, 1995), might have greater expression or increased function in NK1R-/- mice. This nerve terminal protein plays a critical role in the molecular cascade that couples neuronal excitation and transmitter release / secretion (Söllner *et al*, 1993a, 1993b; Bai *et al*, 2004). The finding that neurotransmission can be blocked by botulinum neurotoxins A and E, which specifically

recognize and cleave SNAP-25, provides additional evidence for the involvement of SNAP-25 in vesicle fusion (Blasi *et al*, 1993; Schiavo *et al*, 1993). In NK1R-/- mice, increased expression / function of SNAP-25 might give rise to a more efficient release process. Because of this 'modified' release cascade in NK1R-/- mice, their NA release from noradrenergic terminals might take place more readily, compared with the wild-type. This might explain why comparable NA responses to the two successive K^+ pulses were observed only in the mutants. It is noteworthy that in the past decade, SNAP-25 has gathered special attention in ADHD. This is because the ADHD model, the coloboma mouse, has mutations on the SNAP-25 gene (Hess *et al*, 1996). Moreover, a polymorphism for the SNAP-25 gene has been linked with ADHD (Mill *et al*, 2002; Feng *et al*, 2005). This is consistent with later results which showed the linkage between NK1R-/- mice and ADHD.

- **The effects of Ca^{2+} removal from the perfusion medium (i.e. K^+ / Ca^{2+}-free)**

The data revealed that removal of Ca^{2+} from the perfusion medium diminished the NA response to the 80 mM K^+ pulse in NK1R-/- mice. However, such an effect of Ca^{2+}-depletion was not clear in NK1R+/+ mice, as the NA response to the *second* K^+ pulse did not differ from that to the *second* Ca^{2+}-free pulse. Two possible explanations are proposed here:

Hypothesis 1: the apparent lack of an effect of Ca^{2+}-depletion in NK1R+/+ mice could be due to the existence of **residual Ca^{2+} ions** in extracellular space (possibly diffused from adjacent sites), despite the removal of Ca^{2+} ions from the perfusion medium during the 'Ca^{2+}-free' pulse.

Hypothesis 2: the finding that the NA response of NK1R+/+ mice to the *second* K^+ pulse did not differ from that to the *second* Ca^{2+}-free pulse might not reflect a lack of effect of Ca^{2+}-depletion. It might just be that **the increase in NA efflux by the *second* K^+ pulse was so small** that it did not differ from the NA efflux after removal of Ca^{2+} ions.

It is important to show that NA release in the PFC of NK1R+/+ mice is Ca^{2+}-dependent, therefore the experiments described in Section 3.4.2 and Section 3.4.3 were performed, using new batches of wild-types.

3.5.2. Double-pulse experiments (with EGTA)--in NK1R+/+ mice only

This experiment was carried out to test Hypothesis 1. The Ca^{2+} chelator, EGTA, was co-infused during the Ca^{2+}-free pulse to ensure the minimal presence of any extracellular Ca^{2+} ions. However, the NA response to the *second* K^+ pulse still did not differ from that to the *second* Ca^{2+}-free pulse (in which EGTA was included). This therefore suggested that the failure to observe an effect of Ca^{2+}-depletion in NK1R+/+ mice is unlikely to be due to the presence of residual Ca^{2+} ions at extracellular sites.

3.5.3. Single-pulse experiments (with EGTA)--in NK1R+/+ mice only

To test Hypothesis 2, in a new group of NK1R+/+ mice, a single-pulse was given, in which Ca^{2+} was removed (and EGTA added) without any prior K^+ challenge. The results showed a clear effect of Ca^{2+} depletion in these animals, as their NA response to removal of Ca^{2+} was significantly lower compared with that to the single, time-matched 80 mM $[K^+]_o$ pulse. Hence, these findings favour Hypothesis 2 and suggested that NA release in the PFC of NK1R+/+ mice is Ca^{2+}-dependent, as with NK1R-/- mice. Thus, microdialysis is validated: NA is derived from impulse-dependent release. However, this Ca^{2+}-dependency in the wild-type might have been masked by their lack of ability to keep up the NA response to the *second* K^+ pulse.

To summarize: NA efflux in the PFC of both NK1R+/+ and NK1R-/- mice is Ca^{2+}-dependent. This is in line with the well established role of this ion in impulse-evoked transmitter release (Katz & Miledi, 1965; Hubbard *et al*, 1968; Augustine *et al*, 1987). Nevertheless, there is a clear difference in regulation of impulse-evoked NA release in the two genotypes. NK1R+/+ mice did *not* display a sustained NA response to successive K^+ stimulations, possibly due to: (i) activation of presynaptic α_2-autoreceptors, which reduce impulse-dependent NA release; (ii) exhaustion of transmitter stores and / or a less efficient excitation-release process, compared with the knockout. On the other hand, NK1R-/- mice seemed to be more sensitive to Ca^{2+}-depletion than the wild-type. This is possibly because, in the mutants, NA release is increased (as their α_2-autoreceptors are desensitized), which leads to a greater demand for Ca^{2+} in order to keep up the release process.

3.6. Summary

- **Similarity in NK1R+/+ and NK1R-/- mice:**
  - NA efflux in the PFC of both genotypes is Ca^{2+}-dependent.

- **Differences in NK1R+/+ and NK1R-/- mice:**
  - NK1R-/-, but not NK1R+/+, mice showed a sustained NA response to the two successive K^+ stimulations.

    This might be due to:
    1. NA release is increased in NK1R-/- mice, as their feedback inhibition by α_2-adrenoreceptors is impaired.
    2. The mutants might possess a larger releasable pool of NA than NK1R+/+ mice.
    3. The mutants might have a more efficient excitation-release coupling than NK1R+/+ mice.

  - The effect of Ca^{2+}-depletion on NA efflux in NK1R+/+ mice was only evident when removal of Ca^{2+} was performed *without* prior K^+ stimulation.
    This seemed to be because that the effects of removal of Ca^{2+} in these animals are masked by the small NA response to the *second* K^+ challenge (in the double-pulse experiments).

  - NK1R-/- mice are more vulnerable to Ca^{2+} depletion than NK1R+/+ mice.
    NA release is greater in NK1R-/- mice, due to desensitization of their α_2-autoreceptors in the LC. This might therefore increase the sensitivity of the mutants to depletion of Ca^{2+} in order to keep up with their NA release.

Chapter 4. Indices of abnormal central noradrenaline and dopamine transmission in NK1R-/- mice

4.1. Introduction

Previous studies showed behavioural abnormalities of NK1R-/- mice: they are hyperactive in the light / dark exploration box (LDEB) (Herpfer *et al*, 2005; Fisher *et al*, 2007), and they do not respond to the rewarding effects of *d*-AMP (Murtra *et al*, 2000a) or morphine (Murtra *et al*, 2000a, b; Ripley *et al*, 2002; Gadd *et al*, 2003) in the conditioned place preference (CPP) test. It is thought that the motor arousing and rewarding effects of psychostimulants, like *d*-AMP, are mediated by the drug-induced increase in central catecholamine transmission (Darracq *et al*, 1998; Sellings & Clarke, 2003; Ventura *et al*, 2005). However, regulation of NA transmission by α_2-adrenoceptors is impaired in NK1R-/- mice (Herpfer *et al*, 2005; Fisher *et al*, 2007). Therefore, the **first aim** of this series of experiments was to investigate whether an abnormal NA response to local infusion of *d*-AMP in the PFC of the mutants (if any) could underpin their atypical behavioural response to the drug, using *in vivo* microdialysis.

A key variable that is likely to influence the effect of *d*-AMP on PFC NA efflux, is the action of presynaptic α_2-adrenoceptors. In the PFC, ***α_2-adrenoceptor-mediated compensatory reduction of impulse-dependent release*** can mask a ***d-AMP-induced release of NA*** (Géranton *et al*, 2003). In rats, at least, this compensatory reduction is only evident at low *d*-AMP concentrations. At higher concentrations, however, *d*-AMP-induced impulse-independent NA release predominates and masks any autoinhibition effect of α_2-autoreceptors. This is evident by the microdialysis study from this lab: by infusing *d*-AMP (10 µM and 100 µM) locally into the PFC of freely-moving rats, Géranton *et al* (2003) showed that NA efflux in this brain region was dose-dependently augmented. However, this dose-dependency was abolished by pretreatment with the selective α_2-adrenoceptor antagonist, atipamezole, which increased the NA response to the 10 µM, but not 100 µM, *d*-AMP.

Given that function of the α_2-autoreceptors in the LC is impaired in NK1R-/- mice (Herpfer *et al*, 2005; Fisher *et al*, 2007), their abnormal NA transmission after the *d*-AMP treatment (if any) might be attributed to a lack of functional α_2-autoreceptors. To find out whether this could be the case, the **second aim** was to test the effect of *d*-AMP on PFC NA efflux in NK1R+/+ and NK1R-/- mice, pretreated with an α_2-adrenoceptor antagonist, atipamezole (K_i values = 1 nM; Bylund *et al*, 1988; Harrison *et al*, 1991). This antagonist was chosen because systemic

administration of this compound has been shown to augment the cortical NA response to *d*-AMP in freely-moving rats (Wortley *et al*, 1999b; Géranton *et al*, 2003).

Because differences in the NA response to *d*-AMP were evident in these experiments, I went onto compare the locomotor response to this psychostimulant in NK1R+/+ and NK1R-/- mice (see Chapter 5). The results were very interesting, and prompted us to investigate the behavioural effect of another psychostimulant, methylphenidate (MPH). This, in the end, also revealed robust genotype differences (also see Chapter 5). As an uptake blocker, MPH enhances both central NA and DA transmission (Hurd & Ungersted, 1989; Kuczenski & Segal, 1997). For this reason, we were curious to find out whether the atypical behavioural response of NK1R-/- mice to MPH could be associated with their abnormal catecholaminergic response to this drug. Thus, the **final aim** of the experiments described in this chapter is to assess the NA and DA responses to local infusion of MPH in the PFC of freely-moving NK1R+/+ and NK1R-/- mice.

4.2. Aim

***In vivo* microdialysis was used to:**

- **Characterize the NA response to local infusion of *d*-AMP in the PFC of freely-moving NK1R+/+ and NK1R-/- mice**
- **Compare the NA response to local infusion of *d*-AMP in the PFC of freely-moving NK1R+/+ and NK1R-/- mice, pretreated systemically with the α_2-adrenoceptor antagonist, atipamezole.**

} ***d*-AMP**

- **Investigate the NA response to local infusion of MPH in the PFC of freely-moving NK1R+/+ and NK1R-/- mice**
- **Investigate the DA response to local infusion of MPH the PFC of freely-moving NK1R+/+ and NK1R-/- mice**

} **MPH**

4.3. Protocols

4.3.1. The NA response to local infusion of *d*-AMP in the PFC

On the day of dialysis, after obtaining at least 3 consecutive stable basal NA samples, *d*-AMP was locally infused by retrodialysis into the PFC of NK1R+/+ and NK1R-/- mice, at 10 μM and then 100 μM (dissolved in Ringer's solution)----Note that the actual drug concentration outside the microdialysis probe would be about 10% that within the probe and would decline with increasing distance (see Section 2.3.4.1). Perfusion of each drug concentration lasted for 120 min, and between the two concentrations, Ringer's solution was perfused for 60 min (see Fig 4.1):

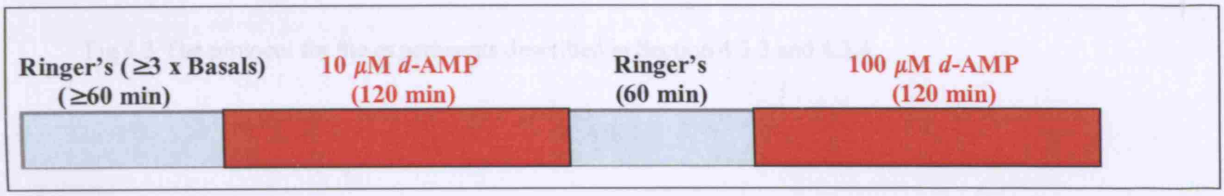


Fig 4.1 The protocol for the experiments described in Section 4.3.1.

4.3.2. The effects of *d*-AMP in mice pretreated with an α_2-antagonist, atipamezole

Another batch of NK1R+/+ and NK1R-/- mice were pretreated intraperitoneally with the selective α_2-antagonist, atipamezole (Pfizer) at a dose of 1 mg/kg (dissolved in 0.9% saline, given at a volume of 10 ml/kg). This dose was used, as it is effective in rats (Géranton *et al*, 2003). The pretreatment was performed 120 min prior to infusion of *d*-AMP in order to allow any increase in NA efflux following the stress of injection to return to baseline before *d*-AMP was administered (see Fig 4.2). Previous evidence from this lab showed that, in rats, the effects of atipamezole can last for at least 6 hour post administration (Wortley *et al*, 1999b).

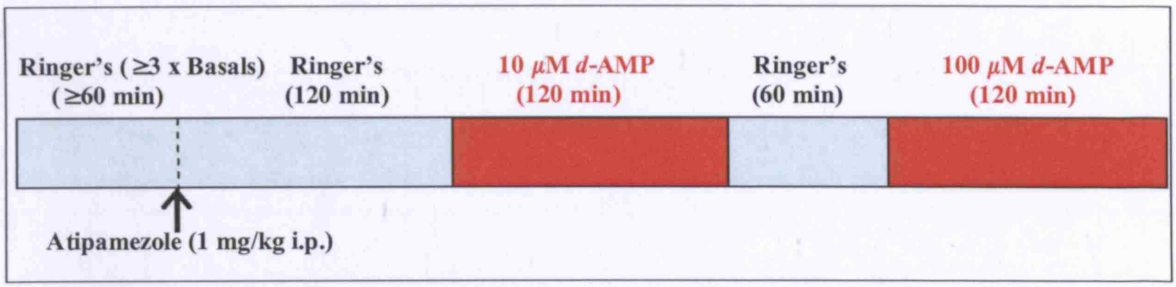


Fig 4.2 The protocol for the experiments described in Section 4.3.2.

4.3.3. (and 4.3.4.) The NA (and DA) response to local infusion of MPH in the PFC

New batches of NK1R+/+ and NK1R-/- were used. The protocol (see Fig 4.3) was the same as described in Section 4.3.1, but substituting MPH for *d*-AMP.

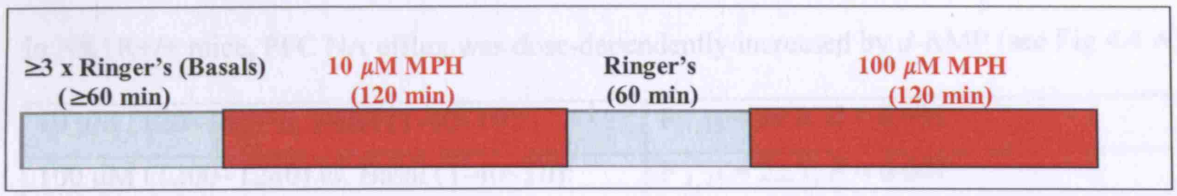


Fig 4.3 The protocol for the experiments described in Section 4.3.3 and 4.3.4.

4.4. Results

4.4.1. The NA response to local infusion of *d*-AMP in the PFC

Statistics were performed using the raw data (see Fig 4.4 A) unless otherwise stated.

In NK1R+/+ mice, PFC NA efflux was dose-dependently increased by *d*-AMP (see Fig 4.4 A):

10 μM (T20~T60) *vs.* Basal (T-40~T0):	$F_{1,13} = 39.8$, $P < 0.001$
100 μM (T200~T240) *vs.* Basal (T-40~T0):	$F_{1,13} = 22.7$, $P < 0.001$
10 μM (T20~T80) *vs.* 100 μM (T200~T260):	$F_{1,13} = 4.4$, $P < 0.05$

In NK1R-/- mice, NA efflux in the PFC was augmented by 10 μM *d*-AMP. Despite the fact that the apparent increase at 100 μM did not reach the statistical criterion (see $^{+}$ below), the NA response did not differ at the two drug concentrations (see Fig 4.4 A):

10 μM (T20~T60) *vs.* Basal (T-40~T0):	$F_{1,11} = 15.8$, $P < 0.01$
100 μM (T200~T240) *vs.* Basal (T-40~T0):	Did not reach significance ($P = 0.129$) $^{+}$

At both *d*-AMP concentrations, the PFC NA response was weaker in NK1R-/- than in NK1R+/+ mice. Specifically, at 100 μM *d*-AMP, the *peak NA efflux* was 1.3-fold smaller in NK1R-/- (15.8 ± 1.2 fmol / 20min) than in NK1R+/+ mice (20.2 ± 1.8 fmol / 20min). At 10 μM, the *peak NA efflux* did not differ in the two genotypes, but a *main effect of genotype* was still observed over the perfusion period T40~T140. These genotype differences were rather small and were evident only when comparing the net changes in NA efflux (see Fig 4.4 B):

Main effect of 'Genotype'----At 10 μM (T40~T140):	$F_{1,24} = 5.3$, $P < 0.05$
Main effect of 'Genotype'----At 100 μM (T200~T300):	$F_{1,24} = 5.6$, $P < 0.05$

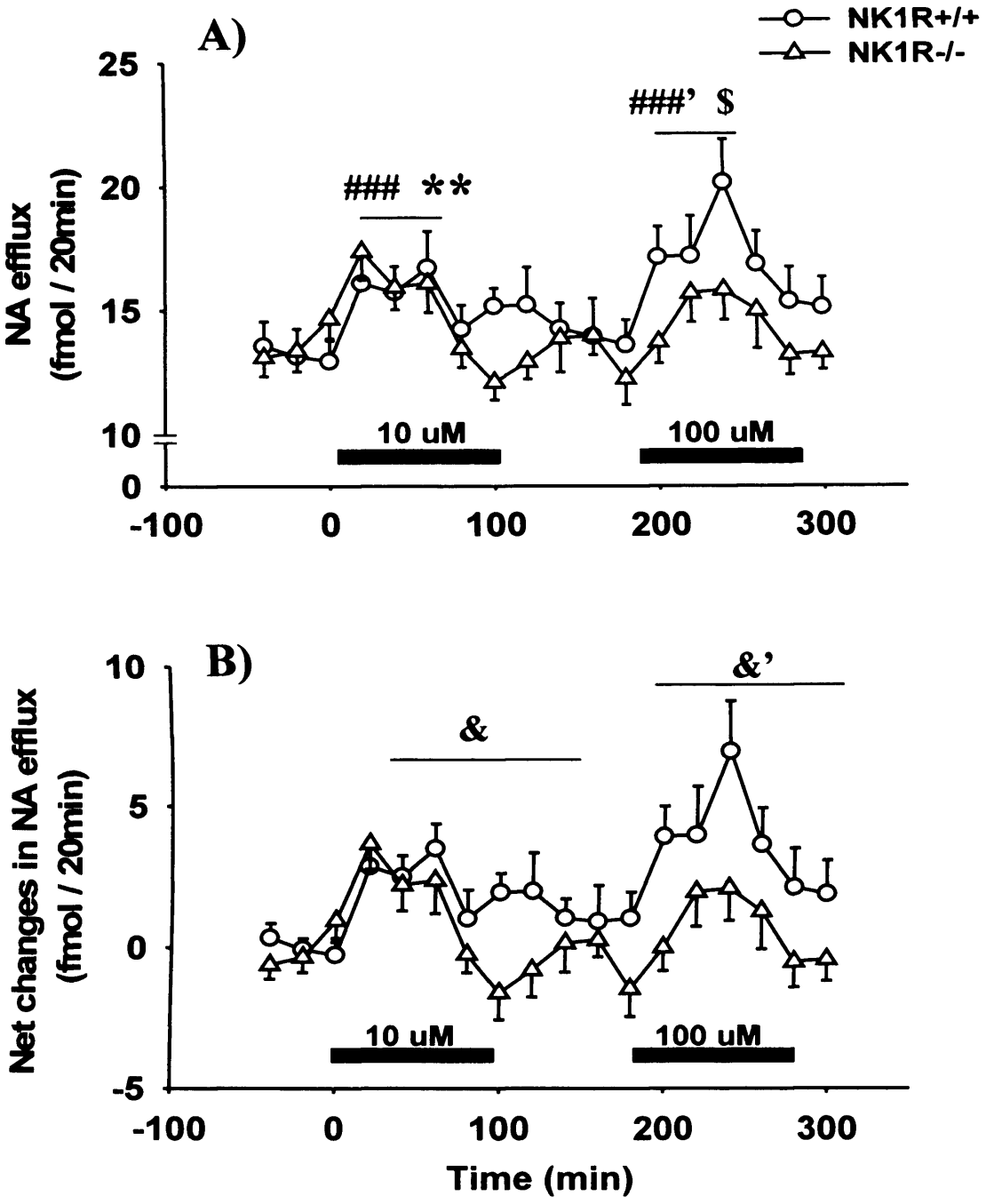


Fig 4.4 **A)** Raw data and **B)** the net changes showing the NA response to local infusion of *d*-AMP in the PFC of NK1R+/+ and NK1R-/- mice (N = 12~13/group).

A) NK1R+/+ mice----10 μM *vs.* basals: ### $P < 0.001$; 100 μM *vs.* basals: ###' $P < 0.001$;
10 μM *vs.* 100 μM: $ $P < 0.05$.

NK1R-/- mice----10 μM *vs.* basals: ** $P < 0.01$.

B) NK1R+/+ *vs.* NK1R-/- mice----at 10 μM: & $P < 0.05$; at 100 μM: &' $P < 0.05$.

4.4.2. The effects of *d*-AMP in mice pretreated with an α_2-antagonist, atipamezole

Statistics were performed using the raw data (see Fig 4.5 A) unless otherwise stated.

In the atipamezole-pretreated NK1R+/+ mice, NA efflux in the PFC was augmented by both concentrations of *d*-AMP. The extent to which NA efflux was enhanced did not differ at the two drug concentrations (see Fig 4.5 A):

10 μM (T140~T180) *vs.* Basal (T-40~T0):	$F_{1,13} = 14.2$, $P < 0.01$
100 μM (T320~T360) *vs.* Basal (T-40~T0):	$F_{1,13} = 22.0$, $P < 0.001$

d-AMP also augmented the PFC NA efflux in the atipamezole-pretreated NK1R-/- mice at both drug concentrations. The NA response did not differ at the two concentrations (see Fig 4.5 A):

10 μM (T140~T180) *vs.* Basal (T-40~T0):	$F_{1,11} = 10.0$, $P < 0.01$
100 μM (T320~T360) *vs.* Basal (T-40~T0):	$F_{1,12} = 9.9$, $P < 0.01$

At 10 μM *d*-AMP, the NA response was greater in NK1R+/+ than in NK1R-/- mice. This genotype difference was, again, evident only when comparing the net changes in NA efflux (see Fig 4.5 B). However, the NA response to 100 μM *d*-AMP did not differ in the two genotypes.

At 10 μM (T160~T260):	$F_{1,25} = 5.5$, $P < 0.05$
At 100 μM (T320~T420):	Not significant

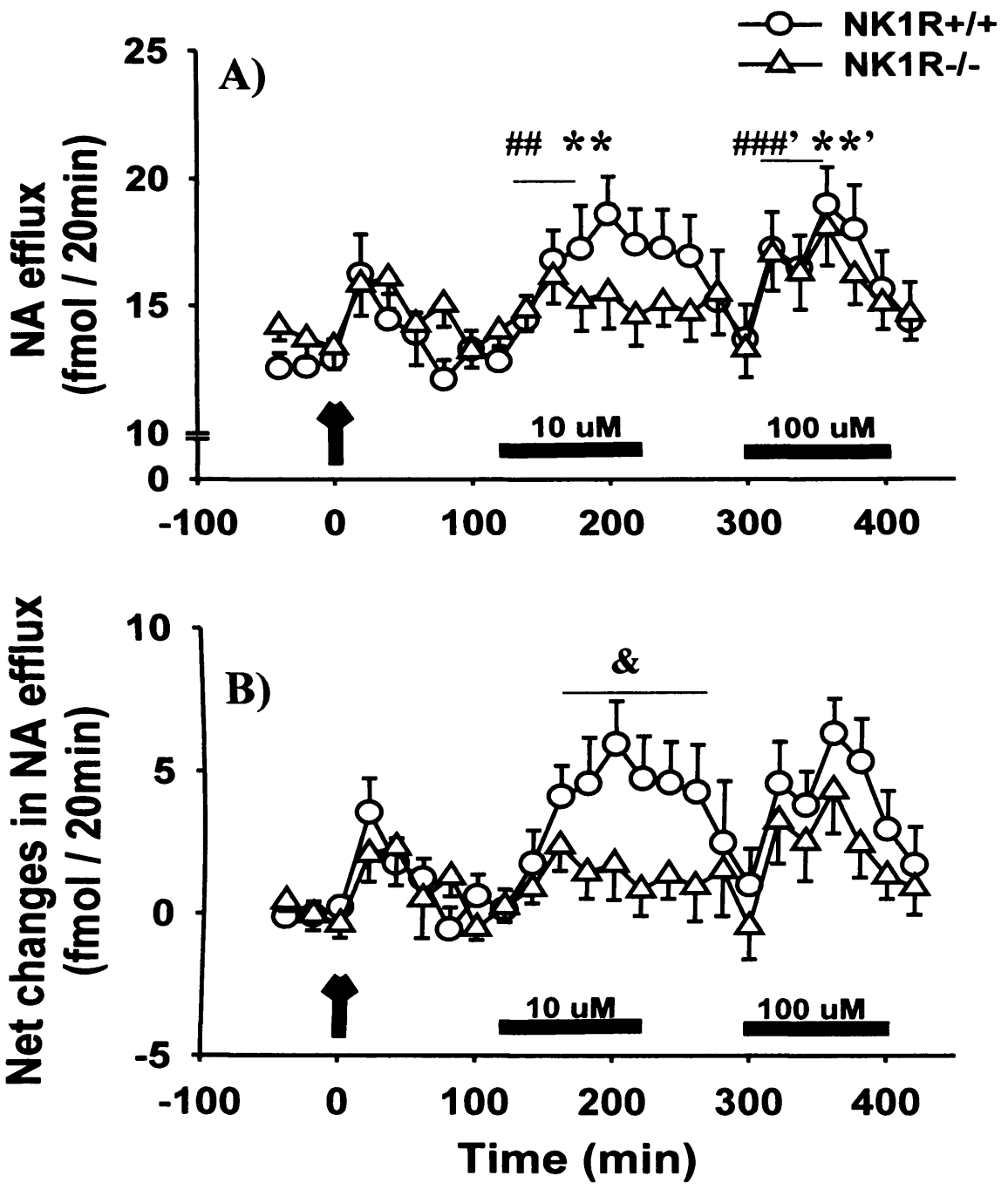


Fig 4.5 **A)** Raw data and **B)** the net changes showing the NA response to local infusion of *d*-AMP in the PFC of NK1R+/+ and NK1R-/- mice pretreated with the α_2-adrenoceptor antagonist, atipamezole (N=12~13/group). The arrow corresponds to dosing with atipamezole.

A) NK1R+/+ mice----10 μM *vs.* basals: ## $P < 0.01$; 100 μM *vs.* basals: ###' $P < 0.001$.

NK1R-/- mice----10 μM *vs.* basals: ** $P < 0.01$; 100 μM *vs.* basals: **' $P < 0.01$.

B) NK1R+/+ *vs.* NK1R-/- mice----at 10 μM: & $P < 0.05$.

4.4.3. The NA response to local infusion of MPH in the PFC

All statistics were performed using the raw data (see Fig 4.6 A).

MPH increased the NA response in the PFC of both NK1R+/+ and NK1R-/- mice at both concentrations.

NK1R+/+ mice (see Fig 4.6 A):

10 μM (T20~T60) *vs.* Basal (T-40~T0):	$F_{1,5} = 26.4$, $P < 0.01$
100 μM (T200~T240) *vs.* Basal (T-40~T0):	$F_{1,5} = 14.6$, $P < 0.05$

NK1R-/- mice (see Fig 4.6 A):

10 μM (T20~T60) *vs.* Basal (T-40~T0):	$F_{1,5} = 29.3$, $P < 0.01$
100 μM (T200~T240) *vs.* Basal (T-40~T0):	$F_{1,5} = 43.8$, $P < 0.001$

The NA response to MPH did not differ at the two drug concentrations, and did not differ in the two genotypes.

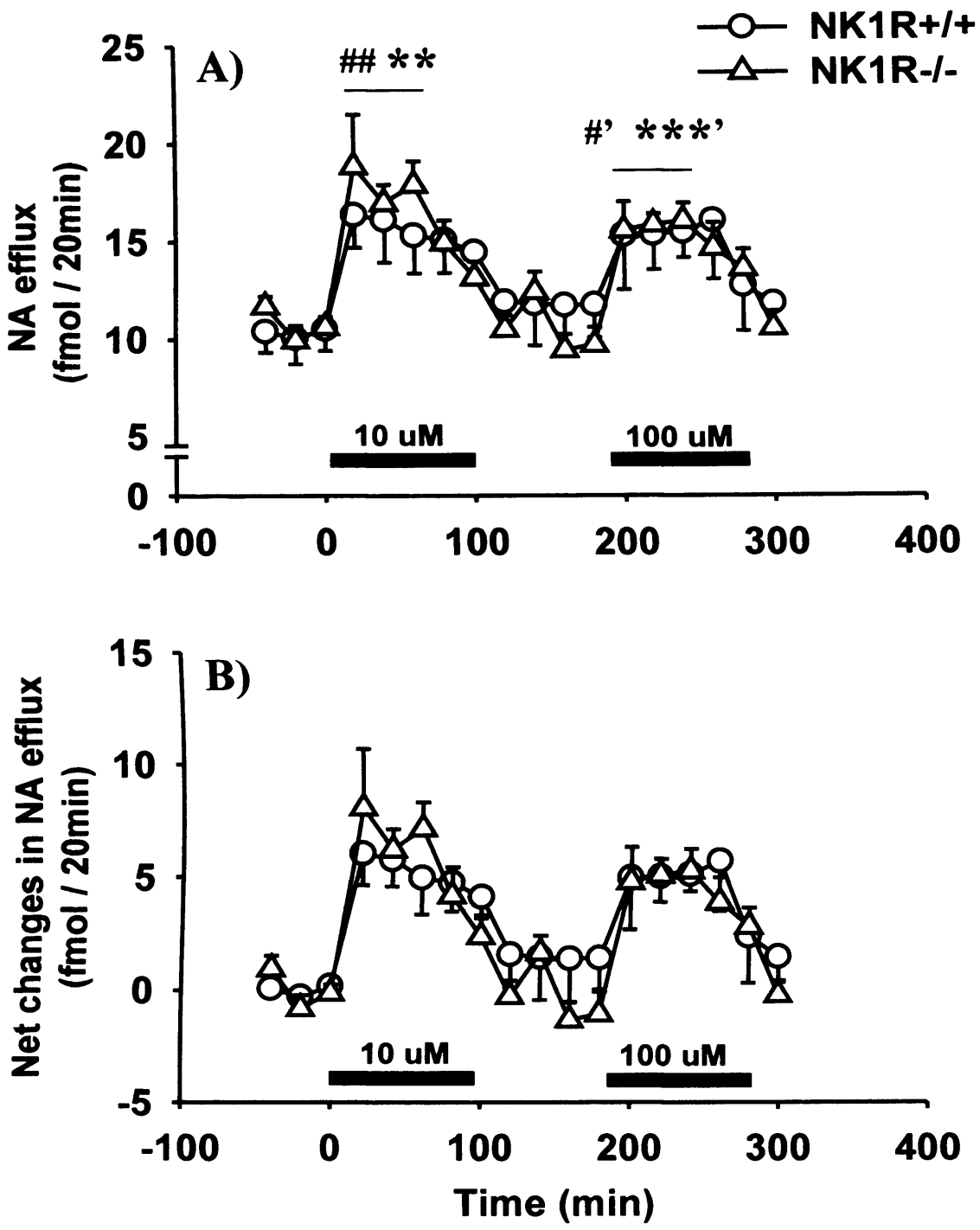


Fig 4.6 **A)** Raw data and **B)** the net changes showing the NA response to local infusion of MPH in the PFC of NK1R+/+ and NK1R-/- mice (N = 6/group).

NK1R+/+ mice----10 μM *vs.* basals: ## $P < 0.01$; 100 μM *vs.* basals: #' $P < 0.05$.

NK1R-/- mice----10 μM *vs.* basals: ** $P < 0.01$; 100 μM *vs.* basals: ***' $P < 0.001$.

4.4.4. The DA response to local infusion of MPH in the PFC

Statistics were performed using the raw data (see Fig 4.7 A) unless otherwise stated.

Basal DA efflux was 1.8-fold greater in NK1R+/+ (85.5 ± 13.2 fmol / 20min) than in NK1R-/- mice (47.0 ± 2.9 fmol / 20min) (see Fig 4.7 A). This genotype difference in basal DA efflux in the PFC was also evident in the experiments described in Chapter 6.

Main effect of 'Genotype'----At T-40~T0:	$F_{1,8} = 8.3$, $P < 0.05$

In NK1R+/+ mice, the apparent increase in PFC DA efflux by 10 μM MPH did not reach the statistical criterion (see $^{+}$ below). At 100 μM, MPH increased the PFC DA response (main effect of Bin: see ^ below), but this increase was *transient* ('Bin*Time' interaction: see ^^ below) (see Fig 4.7 A):

10 μM (T20~T40) *vs.* Basal (T-20~T0):	Did not reach significance ($P = 0.112$) $^{+}$
100 μM (T200~T220) *vs.* Basal (T-20~T0):	$F_{1,4} = 23.5$, $P < 0.01$ ^
100 μM (T200~T220) *vs.* Basal (T-20~T0):	$F_{1,4} = 14.6$, $P < 0.05$ ^^

In NK1R-/- mice, MPH caused a small increase in cortical DA efflux (+40%) at 10 μM, but not at 100 μM (see Fig 4.7 A). Moreover, when comparing the net changes in DA efflux in the two genotypes, the DA response to 10 μM MPH was greater in NK1R-/- mice than in NK1R+/+ mice (see Fig 4.7 B):

10 μM (T20~T40) *vs.* Basal (T-20~T0):	$F_{1,4} = 14.6$, $P < 0.05$
Main effect of 'Genotype'----At 10 μM (T40~T140):	$F_{1,8} = 6.7$, $P < 0.05$

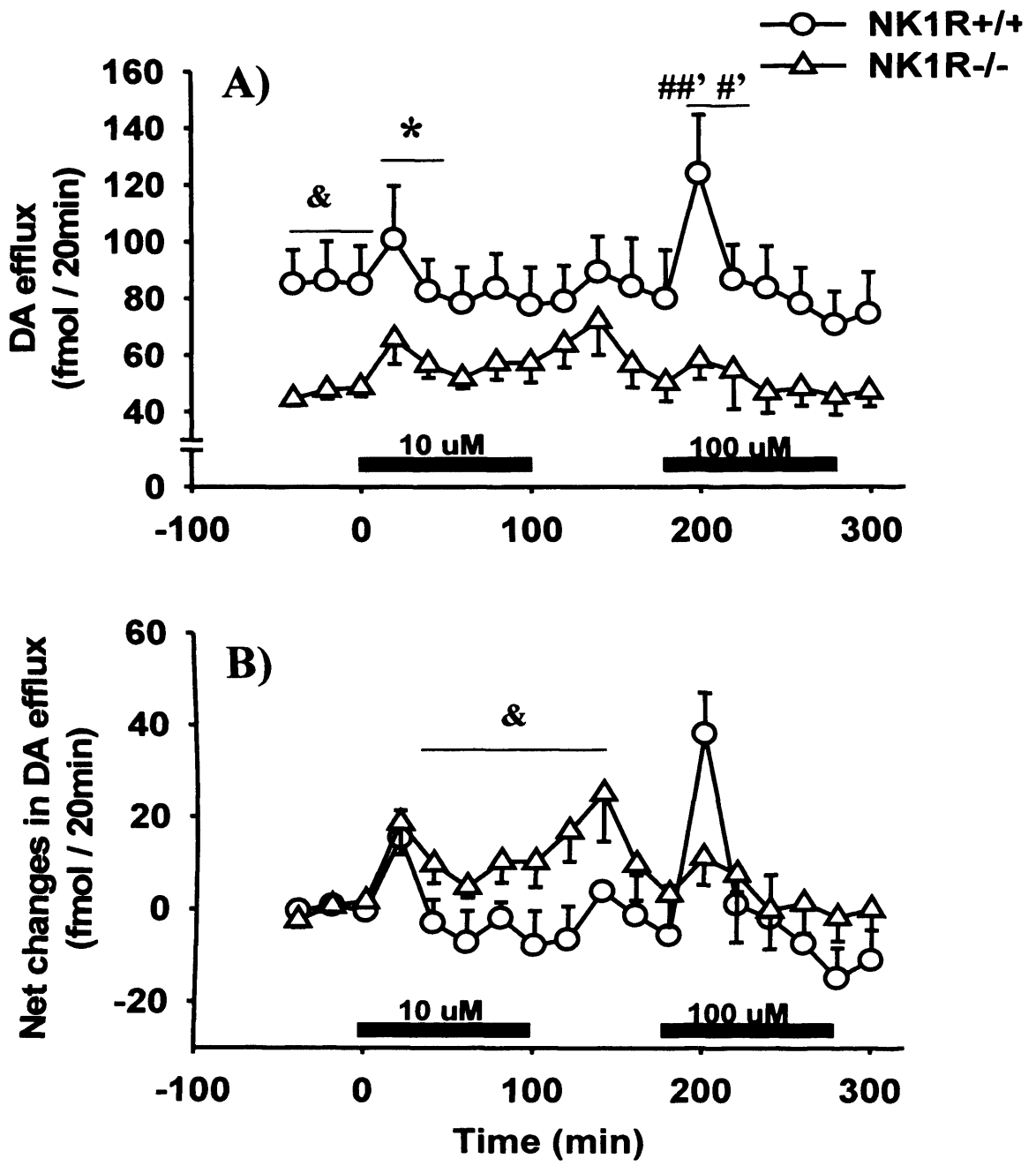


Fig 4.7 **A)** Raw data and **B)** the net changes showing the DA response to local infusion of MPH in the PFC of NK1R+/+ and NK1R-/- mice (N = 5/group).

A) NK1R+/+ *vs.* NK1R-/- mice----Basal DA difference: & $P < 0.05$.

NK1R+/+ mice----100 μM *vs.* basals: main effect of Bin: ##' $P < 0.01$.

'Bin*Time' interaction: #' $P < 0.05$.

NK1R-/- mice----10 μM *vs.* basals: * $P < 0.05$.

B) NK1R+/+ *vs.* NK1R-/- mice----10 μM (T40~T140): & $P < 0.05$.

4.5. DISCUSSION

The findings reported in this chapter revealed abnormal PFC NA and DA responses of NK1R-/- mice to local infusion of the psychostimulants, *d*-AMP and MPH.

4.5.1. The NA response to local infusion of *d*-AMP in the PFC

NA efflux in the PFC of NK1R+/+ mice was dose-dependently augmented by local infusion of *d*-AMP, whereas the increase in NK1R-/- mice did not differ at the two drug concentrations. Further, the NA response to *d*-AMP was weaker in NK1R-/- than in NK1R+/+ mice at both drug concentrations: at 100 μM, the peak efflux of the mutants was smaller; at 10 μM, their peak efflux did not differ from that of the wild-type, but the response was switched off more rapidly, despite the continuous perfusion.

The PFC NA response in NK1R+/+ mice to infusion of *d*-AMP is similar to that seen in rats (Géranton *et al*, 2003b). Hence, the current findings are consistent with the theory that, at low drug concentrations, the PFC NA response to *d*-AMP is constrained by activation of α_2-autoreceptors, which cause a compensatory reduction in impulse-evoked NA release. At the higher drug concentrations, however, NA efflux in the PFC is derived mainly from the psychostimulant-induced impulse-independent release, which the compensatory reduction of impulse-evoked release by α_2-autoreceptors could no longer mask.

In contrast, in NK1R-/- mice, the NA response did not differ at 10 μM and 100 μM *d*-AMP, and the response was weaker than that of the wild-type at both concentrations. Based on these results, it is possible that some factors are switching off the PFC NA response to *d*-AMP more rapidly in NK1R-/- than in NK1R+/+ mice, or there could be less *d*-AMP-induced impulse-independent NA release in the mutants. Although the underlying explanation is yet to be found out, several possibilities are proposed here:

First, the LC NA firing can be reduced by the 5-HT system (*via* the excitatory 5-HT_{2A} receptors located on the inhibitory GABA neurones that project to the LC, as evident by extracellular recording studies: Chiang & Aston-Jones, 1993; Szabo & Blier, 2001). This is supported by later microdialysis study from our group, which showed that the 5-HT synthesis inhibitor, para-chlorophenylalanine (pCPA) augmented the amplitude of, and prolonged, the PFC NA response to local infusion of *d*-AMP (10 μM) in rats (Géranton *et al*, 2004). The weaker NA response to *d*-AMP in NK1R-/- mice could be due to greater 5-HT inhibition of the NA system, as the mutants display increased central 5-HT release (Froger *et al*, 2001). *Secondly*, there could be a compensatory increase in NA uptake in the mutants, given that their NA release is increased (Fisher *et al*, 2007). As the amount of NAT proteins in the PFC did not differ in the two genotypes (Fisher *et al*, 2007), the increased uptake might be mediated by other transporters, such as the organic cation transporters (OCTs; Russ *et al*, 1996; Wu *et al*, 1998; Breidert *et al*, 1998). *Thirdly*, in NK1R-/- mice, the increase in NA release (Herpfer *et al*, 2005; Fisher *et al*, 2007) might lead to a greater competition with *d*-AMP for transport at the uptake site. This could therefore reduce the amount of drug being transported into the cell.

Whether any of the above explanations could explain the abnormal PFC NA response to *d*-AMP in NK1R-/- mice is beyond the scope of this thesis.

4.5.2. The effects of *d*-AMP in mice pretreated with an α_2-antagonist, atipamezole

According to the microdialysis evidence in rats from our lab, activation of α_2-adrenoceptors can mask the increase in PFC NA release caused by local infusion of ***low*** concentrations of *d*-AMP (Géranton *et al*, 2003b). The α_2-adrenoceptors in the LC are desensitized in NK1R-/- mice (Herpfer *et al*, 2005; Fisher *et al*, 2007). Hence, to test whether the difference in function of α_2-adrenoceptors in the two genotypes is the only factor confounding their NA response to *d*-AMP, the effect of local infusion of the drug was tested in NK1R+/+ and NK1R-/- mice pretreated with the α_2-adrenoceptor antagonist, atipamezole.

In the atipamezole-pretreated NK1R+/+ mice, *d*-AMP increased NA efflux in the PFC at both concentrations. This NA response was not dose-dependent, as with the findings in rats (Géranton *et al*, 2003b). Again, the current findings are in agreement with the theory that the α_2-adrenoceptor-mediated autoinhibition occurred at low, but not high, concentrations of *d*-AMP.

In the atipamezole-pretreated NK1R-/- mice, the extent to which NA efflux in the PFC was increased by the two drug concentrations still did not differ. Moreover, in the presence of the α_2-adrenoceptor antagonist, atipamezole, the PFC NA response to 10 µM *d*-AMP was *still* less in NK1R-/- than in NK1R+/+ mice. This suggested that the genotype difference at low drug concentration is not attributed to any difference in α_2-adrenoceptor function that regulates NA release, but to some other abnormalities in NK1R-/- mice. At 100 µM, the cortical NA response to *d*-AMP did not differ in the two genotypes pretreated with atipamezole. This could be that, at high drug concentration, the overwhelming impulse-independent release of NA has masked any genotype difference in NA efflux.

4.5.3. The NA response to local infusion of MPH in the PFC

The results showed that local infusion of MPH increased NA efflux in the PFC of both NK1R+/+ and NK1R-/- mice. This is consistent with the microdialysis findings in rats (Bymaster *et al*, 2002; Kuczenski & Segal, 2002). The increase in NA efflux is likely to be attributed to blockade of the NAT by MPH, as the drug targets the NAT in rodents (Gatley *et al*, 1996) and humans (Ki = 339 nM: Bymaster *et al*, 2002).

The effects of MPH on PFC NA efflux were not dose-related in either genotype. This could be due to a 'ceiling' effect: the membrane-bound NAT might be saturated at 10 μM MPH, hence a higher concentration of 100 μM could not further augment the NA response. Alternatively, at 100 μM, the MPH-induced increase in NA efflux might be masked by high affinity reuptake *via* membrane-bound transporters other than the NAT (e.g. the OCT).

Further, there was no genotype difference in the NA response to MPH at either concentration. This is consistent with the evidence that function of the NAT is not impaired in NK1R-/- mice (Herpfer *et al*, 2005; Fisher *et al*, 2007). Thus, MPH exerted its effects in the same way in the two genotypes. Moreover, this lack of genotype difference in the PFC NA response to MPH is rather interesting, given that the response to *d*-AMP was weaker in NK1R-/- than in NK1R+/+ mice (reported in Section 4.4.1). These results suggested the cortical NA response to *d*-AMP could be due to factor(s) other than, or in addition to, uptake transport. This needs to be confirmed in a fully randomized study. Further, the difference in the genotype response to *d*-AMP and MPH could be due to some factor(s) holding down the NA response of the mutants to the former drug, but not that to the latter one. This inhibitory factor could again be the 5-HT system (as discussed in Section 4.5.1), as 5-HT transmission can be elevated by *d*-AMP (Eshleman *et al*, 1999; Rothman & Bauman, 2003), but not by MPH (Gatley *et al*, 1996; Bymaster *et al*, 2002).

4.5.4. The DA response to local infusion of MPH in the PFC

The results showed that basal PFC DA efflux in NK1R-/- mice was 1.8-fold less than that in the wild-type. This striking genotype difference was also observed in the experiments described in Chapter 6. The lower DA efflux in the PFC of NK1R-/- mice could underlie the apparent hypofrontality of ADHD. Hypofrontality reflects impaired PFC functioning (e.g. in working memory), and is associated with decreased blood flow and activity of the frontal lobe as a result of a decline in DA transmission in this brain area (Weinberger *et al*, 1986; Daniel *et al*, 1989; Matsuo *et al*, 2005). By using imaging techniques, several groups have reported a reduction of PFC activity and hypofunctioning of cortical DA systems in ADHD patients (Zametkin *et al*, 1990; Ernst *et al*, 1998; Rubia *et al*, 1999). The current finding that local infusion of MPH increased DA efflux in NK1R-/- mice at 10 μM is thus consistent with the evidence that psychostimulants relieve hypofrontality (Daniel *et al*, 1991). This strengthens the later conclusion that NK1R-/- mice are a model of ADHD.

Since efflux depends on both release and uptake, lower basal DA efflux in NK1R-/- mice could be a result of ***increased reuptake*** and / or ***reduced release***. Based on the current findings, an increase in reuptake is less likely to explain the DA deficit in the mutants. This is because, in the PFC, uptake of DA occurs mainly *via* the NAT (Carboni *et al*, 1990; Morón *et al*, 2002), whose function does not seem to differ in the two genotypes (Herpfer *et al*, 2005; Fisher *et al*, 2007). Hence, the NAT-mediated PFC DA uptake is unlikely to be abnormal in the mutants. Moreover, if lower PFC DA efflux in NK1R-/- mice was due to greater reuptake, MPH (which blocks the NAT) would then be expected to produce a marked increase in DA efflux in the mutants. However, the results showed a modest DA response to 10 μM MPH, only. This further suggested that lower PFC DA efflux in NK1R-/- mice might not be due to an increase in reuptake.

On the other hand, if reduced release in NK1R-/- mice did explain their lower basal DA efflux in the PFC, then in NK1R+/+ mice (which have normal release), MPH would be expected to elicit a greater DA response. Interestingly, in the wild-type, the increase in cortical DA efflux by MPH was rather *small* and *transient*, even at the higher drug concentration. This again pointed to the possibility that the cortical DA response to MPH is switched off somehow, as in the *d*-AMP studies in mice (see Section 4.5.1) and rats (Géranton *et al*, 2003b). Collectively, whether the

reduced DA efflux in the PFC of NK1R-/- mice is explained by their reduced release, or by any other factor(s), still requires further investigations.

4.6. Summary

- **Local infusion of *d*-AMP dose-dependently increased PFC NA efflux in NK1R+/+ mice, but the NA response was much weaker and not dose-related in NK1R-/- mice.**
  Hence, the PFC NA response of NK1R-/- to *d*-AMP could be held down by some factor(s).

- **In animals pretreated with the α_2-antagonist, atipamezole, the PFC NA response to 10 μM *d*-AMP was still smaller in NK1R-/- than in NK1R+/+ mice.**
  This suggests that this genotype difference might not be due to impaired functioning of α_2-adrenoceptors in NK1R-/- mice.

- **At 100 μM, the NA response did not differ in the two genotypes.**
  This suggests that impulse-independent NA release at high concentrations of *d*-AMP masks any genotype difference.

- **Local infusion of MPH elicited similar NA responses in the PFC of NK1R+/+ and NK1R-/- mice at both drug concentrations.**
  This suggests that the function of the NAT did not differ in the two genotypes.

- **Basal PFC DA efflux was lower in NK1R-/- mice, but their PFC DA response to local infusion of MPH was greater than in NK1R+/+ mice at 10 μM.**
  The PFC DA deficit may echo hypofrontality of ADHD patients, as does its reversal by MPH, which could reflect the therapeutic effect of this drug in ADHD.

  Local infusion of MPH caused a small and transient increase in PFC DA efflux in NK1R+/+ mice at 100 μM, but not 10 μM.
  Thus the DA response of NK1R+/+ mice to MPH might be held down by some factor(s).

In the experiments described in the next chapter, the behavioural response of NK1R-/- mice to *d*-AMP and MPH was compared to that of the wild-type.

Chapter 5. Abnormal behaviour of NK1R-/- mice in response to d-amphetamine and methylphenidate in the light / dark exploration box

5.1. Introduction

Functional ablation of the NK1 receptor gene in 129/Sv X C57BL/6 mice did not alter their behaviour in the open field or the elevated plus-maze tests (De Felipe *et al*, 1998; Murtra *et al*, 2000b; Rupniak *et al*, 2001a). However, it causes robust changes in their behavioural response in the Light / Dark Exploration Box (LDEB; see Chapter 2 for details of the set-up). Using the LDEB paradigm, our laboratory has consistently shown that NK1R-/- mice are **hyperactive** compared with the wild-type, as reflected by their higher scores for ***locomotor activity*** (Herpfer *et al*, 2005; Fisher *et al*, 2007).

NK1R-/- mice also show atypical behavioural response to *d*-AMP: they do not respond to the **rewarding effects** of this psychostimulant in a conditioned place preference (CPP) test (Murtra *et al*, 2000a). This raises the question of whether the **locomotor arousal** induced by this drug is impaired in the mutants, also (see Fig 5.1).

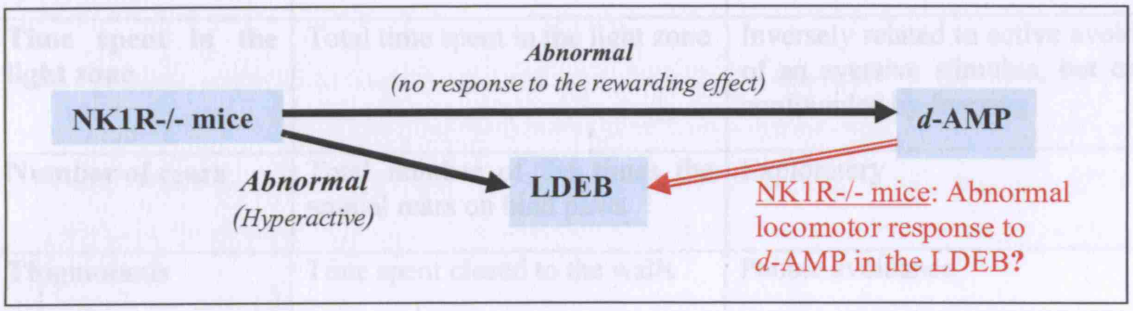


Fig 5.1 Diagrammatic illustration of the relationships between NK1R-/- mice, *d*-AMP and the LDEB test.

The LDEB paradigm was used to test this possibility for several reasons. First, hyperactivity of NK1R-/- mice is evident in this test (Stewart *et al*, 2002; Herpfer *et al*, 2005; Fisher *et al*, 2007). Also, our previous LDEB studies in rats showed that psychologically aversive non-noxious stimuli, such as the transfer to the light zone of the LDEB, increase activation of central noradrenergic neurones without causing physical discomfort (Dalley & Stanford, 1995, McQuade *et al*, 1999). Hence, behaviour of NK1R+/+ and NK1R-/- mice can be compared in the presence of such a non-noxious naturalistic stressor. Another advantage of this test is that it enables the measurement of a wide range of behaviours (see Table 5.1), hence it acts as a broad

Behaviour	Method of Measurement	Indication
Locomotor activity	Number of lines crossed by all four paws of the mouse	A well known and widely accepted parameter in evaluating the arousing effects of stimulants in rodents
Grooming	Mouse licks / scratches its fur and / or washes its face	A common attitude of body care or a territorial behaviour due to a stressful environmental situation and / or behavioural pattern. It is generally recognized as a coping strategy to deal with the stressful situation
Latency to leave the light zone	Time taken to leave the light zone for the first time	Active avoidance of an aversive stimulus (Impulsivity / anxiety-like)
Time to first return to the light zone	Time taken to return the light zone for the first time	Passive avoidance of an aversive stimulus (Impulsivity / anxiety-like)
Number of returns to the light zone	Total number of returns to the light zone	Passive avoidance of an aversive stimulus (Impulsivity / anxiety-like)
Time spent in the light zone	Total time spent in the light zone	Inversely related to active avoidance of an aversive stimulus, but can be confounded by freezing
Number of rears	Total number of the times the animal rears on hind paws	Exploratory
Thigmotaxis	Time spent closed to the walls	Phobic avoidance
Flat-back approach (FBA)	Walking with the body touching the ground	Risk assessment (anxiety-like)
Stretch-attend posture (SAP)	Body stretched forward then retracted to the original position without any forward locomotion	Risk assessment (anxiety-like)
Faecal boli	Number of faecal boli excreted	Indicating the functioning of the gastro-intestinal tract, and possibly also reflecting fear

Table 5.1 Behaviours scored in the LDEB test and their interpretations.

screen, which exposes genotype differences and indicates what behaviours to explore in more detail in future work. In the previous behavioural studies from our lab, several behavioural abnormalities of NK1R-/- mice apart from, or in addition, to their hyperactivity have been revealed in the LDEB. For example, they display lower scores for ***time to first return to the light zone***, ***total time spent in the light zone*** and the risk assessment behaviours ('***flat-back approach***' (***FBA***) and '***stretch-attend postures***' (***SAP***)) (Herpfer *et al*, 2005; Fisher *et al*, 2007). Moreover, the LDEB paradigm can be used in combination with *in vivo* microdialysis, so that we can look for parallel changes in efflux and behaviour. Finally, this test is easy and quick to use, and no prior training of animals is required.

It is important to point out that the LDEB test, first described by Crawley and Goodwin in 1980, is usually used to screen anxiolytic drugs in rodents. However, the LDEB paradigm used in this project followed a different protocol and is not used as a screen of anxiety-like behaviour. Instead, it was used to look for any differences in the behavioural response of NK1R+/+ and NK1R-/- mice to an acute drug challenges.

Using the same protocol as that for the study of *d*-AMP, the behaviour response to MPH was also compared in the two genotypes. This is because the results reported in this chapter showed that *d*-AMP abolished the hyperactivity of NK1R-/- mice, which echoed its therapeutic effects in the treatment of ADHD. Hence, this project went on to test whether MPH, another first-line ADHD medication, would have similar motor effects in the mutants.

Behavioural research using genetically modified animals (e.g. knockouts) is being used a great deal in elucidating the specific role of a single gene product on behaviour. However, genetic modification can cause developmental / compensatory changes that affect the phenotype. Therefore, it is important to identify whether or not any atypical response of the knockout is a result of the absence of the functional targeted receptor. This can be tested using a selective antagonist of the targeted receptor. If the genotype difference is abolished by the antagonist which has no effects in the knockout, it can be confirmed that the abnormality in the mutants is due to the lack of functional targeted receptors.

It should be borne in mind that NK1 receptors in rats / mice differ markedly from those in humans in both pharmacology (Beresford *et al*, 1991; Gitter *et al*, 1991) and CNS localization (Dietl & Palacios, 1991). The compound, GR 205171, would be a suitable NK1R antagonist for the current study, based on its high affinity for the mouse / rat NK1 receptor (see Section 1.3.4). However, this antagonist is not available for research purposes. Hence, the NK1R antagonist, RP 67580, was chosen, which also has a higher affinity for rodents than for humans (*Ki* = 3 nM for rats and 56 nM for humans: Tattersall *et al*, 1996). Moreover, in order to control for any non-specific pharmacological effects of RP 67580, another potent non-peptide NK1R antagonist, L 733060, was also used. L 733060 has a high affinity for human and guinea-pig (Harrison *et al*, 1994; Kramer *et al*, 1998). However, there is evidence that it is effective in C57BL/6 NK1R+/+ mice (Guiard *et al*, 2004), and that it targets mouse NK1 receptors when given intraperitoneally at a dose range of 1.25 ~ 10 mg/kg (Bang *et al*, 2004). Another important reason that RP 67580 and L 733060 were used in the current study is because they penetrate the blood brain barrier into the brain, where central effects of the drugs are exerted. The inactive enantiomer of RP 67580 or L 733060 was not used in this study, since NK1R-/- mice were used as the control.

Interestingly, the genotype difference in locomotor activity is more robust in the light zone of the LDEB than in the dark zone, where mice were habituated to prior to the test. This could be due to the dark zone being not aversive enough to elicit a genotype difference in behaviour. Alternatively, this might be explained by familiarization of animals to the dark zone, which made this environment relatively less stressful compared with the light zone. To test whether any of these two possibilities could underlie the lack of a genotype difference in motor performance in the dark zone of the LDEB, locomotor activity during the 1st 30 min habituation to the dark zone was scored in another batch of NK1R+/+ and NK1R-/- mice.

5.2. Aim

The LDEB paradigm was applied to:

- **Compare the behaviour of NK1R+/+ and NK1R-/- mice, following acute administration of the psychostimulant, *d*-AMP or MPH.**
- **Investigate whether any abnormal behavioural response of NK1R-/- mice was due to disruption of their NK1 receptors, or to developmental / compensatory change(s), using an NK1R antagonist.**
- **Compare locomotor activity of NK1R+/+ and NK1R-/- mice during the 1st 30 min habituation to the dark zone**

5.3. Protocols

5.3.1. The effects of *d*-AMP and MPH on the mouse behaviour in the LDEB

Two LDEB set-ups were used side by side, so that animals were run in pairs. Experiments were carried out every day between 13:00 h and 15:00 h. NK1R+/+ and NK1R-/- mice were randomly assigned for an i.p. injection (10 ml/kg) of either 0.9% saline or *d*-AMP at a dose of 2.5 mg/kg (dissolved in 0.9% saline; Sigma, Poole, U.K). This dose was chosen because it increases locomotor activity in mice (Huotari *et al*, 2004; Ganea *et al*, 2007). At 13:00 h, mice were placed in the dark zone of the LDEB with the partition door closed to allow habituation for a total of 90 min. After the first 60 min, mice received the injection and were then returned to the dark zone for a further 30 min. After the habituation, mice were transferred to the centre of the light zone, facing away from the partition door, which was removed immediately afterwards in order to allow mice to commute freely between the two compartments (see Fig 5.2). Their behaviour (see Table 5.1) was recorded with a Sony Handycam Vision camcorder for 30 min, and later scored 'blind'.

This protocol was repeated with MPH. This drug was also administered at 2.5 mg/kg (i.p.) according to its effectiveness in mice (Zhu *et al*, 2006).

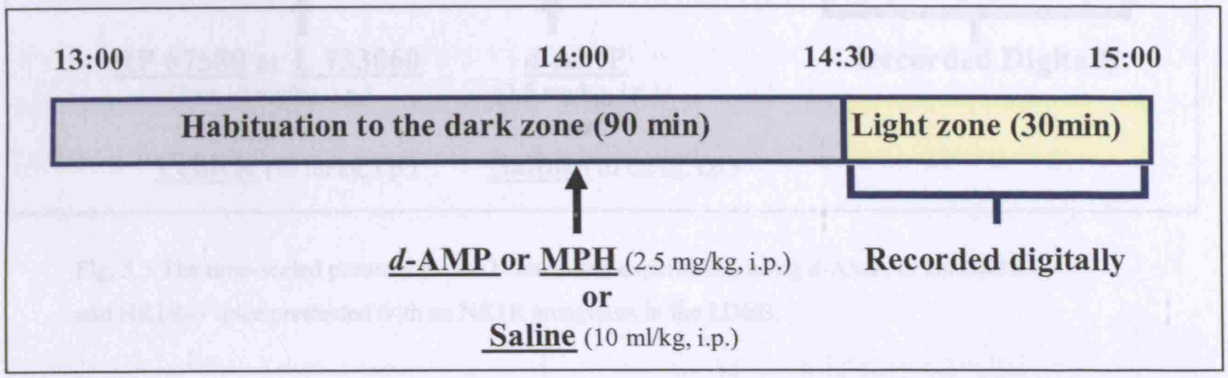


Fig 5.2 The time-scaled protocol for the behavioural experiments, using *d*-AMP or MPH, in the LDEB.

5.3.2. The effects of an NK1R antagonist on the behavioural responses to *d*-AMP

A similar protocol to that described in Section 5.3.1 was carried out (see Fig 5.3). New batches of NK1R+/+ and NK1R-/- mice were randomly assigned to one of the six treatment groups:

Group	Drug 1	Drug 2
1).	Vehicle (Tween 80 + Saline)	Saline
2).	NK1R antagonist (5 mg/kg i.p.)	Saline
3)	NK1R antagonist (10 mg/kg i.p.)	Saline
4)	Vehicle (Tween 80 + Saline)	*d*-AMP (2.5 mg/kg i.p.)
5)	NK1R antagonist (5 mg/kg i.p.)	*d*-AMP (2.5 mg/kg i.p.)
6)	NK1R antagonist (10 mg/kg i.p.)	*d*-AMP (2.5 mg/kg i.p.)

The NK1R antagonists, RP 67580 and L 733060 (Tocris), were dissolved in vehicle (10 μl Tween 80 and final volumes adjusted with 0.9% saline). The antagonists were administered at 5 or 10 mg/kg (i.p.). 5 mg/kg was chosen because Santarelli *et al* (2001) found this dose of RP 67580 to be effective in mice in the elevated plus maze test. The higher dose, 10 mg/kg, was used because, at 1 ~ 10 mg/kg (s.c.), L 733060 has been found to dose-dependently inhibit vocalization induced by transient maternal separation of guinea pig pups (Kramer *et al*, 1998).

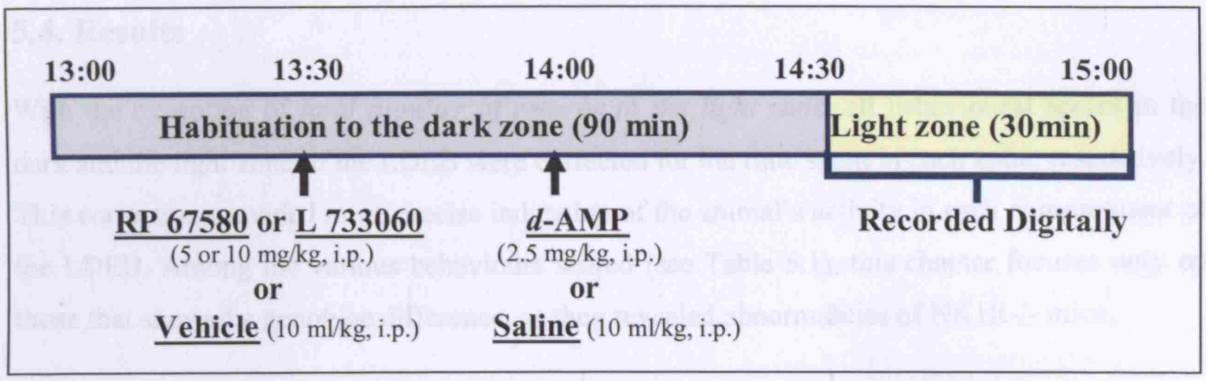


Fig. 5.3 The time-scaled protocol for the behavioural experiment, using *d*-AMP, in the NK1R+/+ and NK1R-/- mice pretreated with an NK1R antagonist in the LDEB.

5.3.3. Locomotor activity during the 1st 30 min habituation to the dark zone

In another batch of NK1R+/+ and NK1R-/- mice, locomotor activity of during the 1st 30 min habituation to the dark zone was recorded and later scored 'blind'.

5.3.4. Statistics

Data were first analysed with multivariate analysis, followed by multifactorial ANOVA (see Section 2.6.2).

For the data reported in Section 5.4.1 and 5.4.2, a 'Genotype*Drug' interaction was taken as the criterion for comparing pairs of data with the Post-Hoc LSD test. It is important to bear in mind that *locomotor activity* and many other behviours could be mutually exclusive, so that the change in the former might mask or explain the latter. Therefore, to eliminate any confounding effects of *locomotor activity* on other behaviours, analysis of covariance (ANCOVA) was performed, using *locomotor activity* as a covariate.

For the data reported in Section 5.4.3, after obtaining a significant 'Genotype*Bin' interaction, locomotor activities of the two genotypes were compared within each time bin, using the Bonferroni t-test.

5.4. Results

With the exception of ***total number of returns to the light zone***, all behavioural scores in the dark and the light zone of the LDEB were corrected for the time spent in each zone, respectively. This correction provided more precise indication of the animal's activity in each compartment of the LDEB. Among the various behaviours scored (see Table 5.1), this chapter focuses only on those that showed a genotype difference, as they revealed abnormalities of NK1R-/- mice.

5.4.1. The effects of *d*-AMP and MPH that depended on genotype

Both drugs increased ***locomotor activity***, ***number of returns to the light zone*** and ***number of rears*** in NK1R+/+ mice, only.

- ***Locomotor activity***

In the light zone of the LDEB, both psychostimulants increased locomotor activity of NK1R+/+ mice. In contrast, the motor response of NK1R-/- mice, after treatment with either drug, did not differ from that of the drug-free wild type (see below for the 'Genotype * Drug' interactions). In fact, *d*-AMP even reduced the hyperactivity of the mutants (LSD test: $P < 0.05$, see Fig 5.4 A):

d-AMP (see Fig 5.4 A)	$F_{1,36} = 14.9$	$P < 0.001$
MPH (see Fig 5.4 B)	$F_{1,43} = 11.1$	$P < 0.01$

In the dark zone, where mice were habituated to prior to the test, the hyperactivity of NK1R-/- mice was not evident during the testing period. *d*-AMP did not affect the motor activity of either genotype in this compartment (see Fig 5.4 C). Nevertheless, MPH again showed a genotype-dependent effect on locomotor activity (see below for the 'Genotype * Drug' interactions): MPH increased it in NK1R+/+ mice (LSD test: $P = 0.001$), but had no effect in NK1R-/- mice (see Fig 5.4 D).

MPH (see Fig 5.4 D)	$F_{1,43} = 6.1$	$P < 0.05$

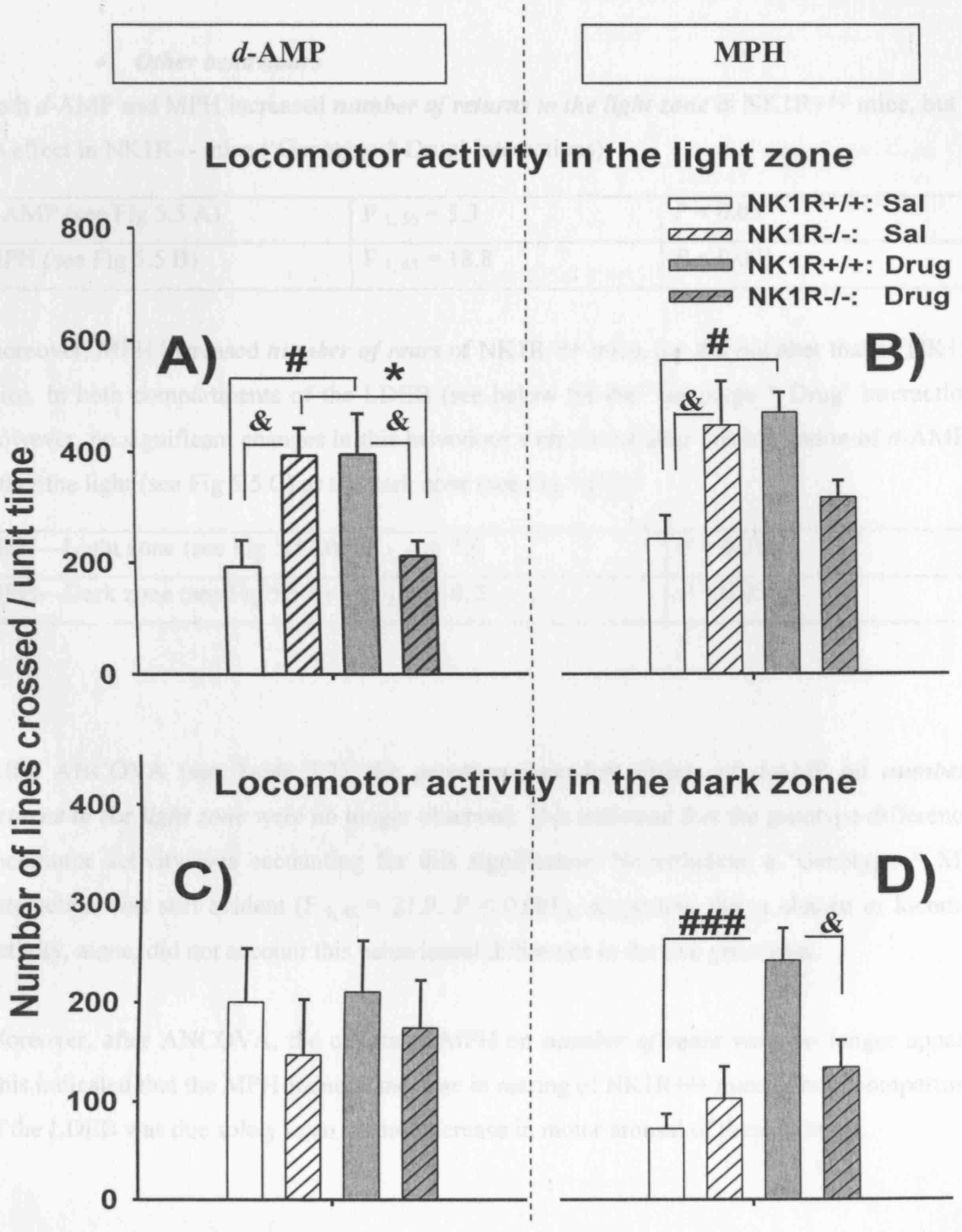


Fig 5.4 The effects of *d*-AMP (graphs A and C; N = 10/group) and MPH (graphs B and D; N = 11~12/group) on locomotor activity of NK1R+/+ and NK1R-/- mice in the light and dark zone of the LDEB. Comparisons between individual groups were performed using the LSD Post-Hoc test, after obtaining significance for a 'Genotype*Drug' interaction. Values are mean ± SEM. Sal: saline. NK1R+/+: # $P < 0.05$, ### $P < 0.001$; NK1R-/-: * $P < 0.05$; NK1R+/+ *vs*. NK1R-/-: & $P < 0.05$.

- ***Other behaviours***

Both *d*-AMP and MPH increased ***number of returns to the light zone*** in NK1R+/+ mice, but had no effect in NK1R-/- mice ('Genotype * Drug' interactions):

d-AMP (see Fig 5.5 A)	$F_{1,36} = 5.3$	$P < 0.05$
MPH (see Fig 5.5 B)	$F_{1,43} = 18.8$	$P < 0.001$

Moreover, MPH increased ***number of rears*** of NK1R+/+ mice, but did not alter that of NK1R-/- mice, in both compartments of the LDEB (see below for the 'Genotype * Drug' interactions). However, no significant changes in this behaviour were found after administration of *d*-AMP, in either the light (see Fig 5.5 C) or the dark zone (see Fig 5.5 E).

MPH—Light zone (see Fig 5.5 D)	$F_{1,43} = 7.2$	$P = 0.01$
MPH—Dark zone (see Fig 5.5 F)	$F_{1,43} = 4.2$	$P < 0.05$

After ANCOVA (see Table 5.2), the genotype-dependent effects of *d*-AMP on ***number of returns to the light zone*** were no longer observed. This indicated that the genotype difference in locomotor activity was accounting for this significance. Nevertheless, a 'Genotype * MPH' interaction was still evident ($F_{1,42} = 21.9$, $P < 0.001$), suggesting that a change in locomotor activity, alone, did not account this behavioural difference in the two genotypes.

Moreover, after ANCOVA, the effects of MPH on ***number of rears*** were no longer apparent. This indicated that the MPH-induced increase in rearing of NK1R+/+ mice in both compartments of the LDEB was due solely to an overall increase in motor arousal of these animals.

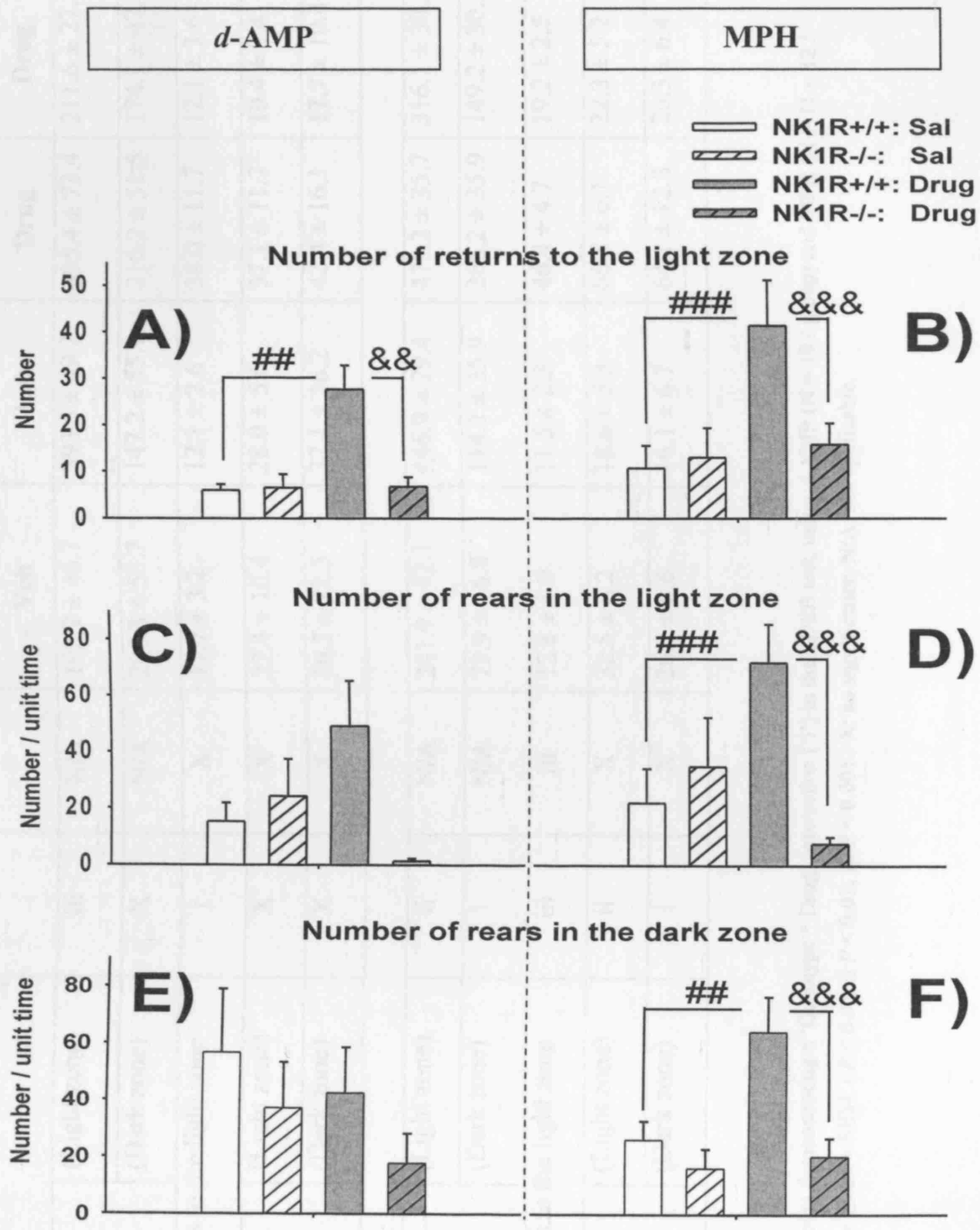


Fig 5.5 The genotype-dependent effects of *d*-AMP (N = 10/group) and MPH (N= 11~12/group) on ***number of returns to the light zone*** and ***number of rears in the light and dark zones***. Comparisons between individual groups were performed using the LSD Post-Hoc test, after obtaining significance for a 'Genotype*Drug' interaction Values are mean ± SEM. Sal: saline. NK1R+/+: ## $P < 0.01$, ### $P < 0.001$; NK1R+/+ *vs*. NK1R-/-: && $P < 0.01$, &&& $P < 0.001$.

Behaviour			2-way		Means ± SEM			
			ANOVA	ANCOVA	NK1R+/+ Veh	NK1R-/- Veh	NK1R+/+ Drug	NK1R-/- Drug
d-AMP	Locomotor activity	(Light zone)	iii	N/A	192.9 ± 46.7	393.5 ± 49.2	395.4 ± 73.4	211.6 ± 27.5
		(Dark zone)	X	N/A	200.3 ± 53.7	147.2 ± 55.7	210.2 ± 51.5	174.1 ± 47.8
	No. of returns to the light zone		i	X	11.7 ± 3.2	12.1 ± 2.6	38.0 ± 11.7	12.1 ± 3.6
	No. of rears	(Light zone)	X	X	27.4 ± 10.4	28.0 ± 5.6	37.1 ± 11.7	10.4 ± 3.4
		(Dark zone)	X	X	56.7 ± 22.3	37.1 ± 16.2	42.4 ± 16.1	17.7 ± 10.4
MPH	Locomotor activity	(Light zone)	ii	N/A	241.9 ± 42.1	446.9 ± 79.4	470.2 ± 35.7	316.1 ± 30.5
		(Dark zone)	i	N/A	79.9 ± 16.8	114.1 ± 35.9	268.2 ± 35.9	149.2 ± 30.2
	No. of returns to the light zone		iii	iii	12.8 ± 1.9	11.5 ± 2.3	46.4 ± 4.7	19.2 ± 2.5
	No. of rears	(Light zone)	ii	X	32.5 ± 6.2	18.6 ± 5.3	66.8 ± 6.1	22.3 ± 5.2
		(Dark zone)	i	X	26.1 ± 6.6	16.1 ± 6.7	64.1 ± 12.3	20.3 ± 6.4

Table 5.2 Behaviours demonstrating a 'Genotype * Drug' interaction ('i') in the LDEB test, using *d*-AMP (N = 10 / group) and MPH (N = 11 ~ 12 / group). Values are mean ± SEM. i $P < 0.05$; ii $P < 0.01$; iii $P < 0.001$. X: no significance. N/A: not applicable.

5.4.2 The effects of NK1R antagonism on the mouse behaviour in the LDEB

The data reported in this section come from the same fully randomized study, but were discussed in two sub-sections for clarity.

Pretreatment with the NK1R antagonist, RP 67580 or L 733060 (5 or 10 mg/kg i.p.), had no effect in NK1R-/- mice, but turned the wild-type into the 'pseudo-knock-out', whose behaviour resembled that of NK1R-/- mice. This was demonstrated in ***locomotor activity*** as well as other behavioural measures in the light zone of the LDEB, which showed a 'Genotype * NK1R antagonist * *d*-AMP' interaction:

Behaviour	**RP 67580**	**L 733060**
Locomotor activity *(in the light zone)*	$F_{2,34} = 7.6$, $P < 0.01$	$F_{2,24} = 4.2$, $P < 0.05$
Number of returns to the light zone	$F_{2,34} = 8.8$, $P < 0.001$	Not significant ($P = 0.329$)
Number of rears *(in the light zone)*	$F_{2,34} = 4.3$, $P < 0.05$	$F_{2,24} = 3.7$, $P < 0.05$
Flat-back approach (FBA) *(in the light zone)*	$F_{2,34} = 20.5$, $P < 0.001$	$F_{2,24} = 3.9$, $P < 0.05$
Stretch-attend posture (SAP) *(in the light zone)*	$F_{2,34} = 4.4$, $P < 0.05$	Not significant ($P = 0.084$)

A significance on the above 3-way ANOVA interaction was taken as the criterion to compare subgroups using 2-way ANOVA (see later). A significant 2-way ANOVA 'Genotype * Drug' interaction was then taken as the criterion for comparing pairs of data with the Post-Hoc LSD test.

In contrast to these behavioural differences in the *light* zone of the LDEB, no difference was found between groups in the *dark* zone, where hyperactivity of NK1R-/- mice was not evident.

- **Effects of NK1R antagonists alone** ***(i.e. in mice which did not receive d-AMP)***

To determine the effects of NK1R antagonists, alone, the groups that received the following treatments were compared:

Group	Drug 1	Drug 2
1).	Vehicle	Saline
2).	NK1R antagonist (5 mg/kg i.p.)	Saline
3)	NK1R antagonist (10 mg/kg i.p.)	Saline

- ***Locomotor activity***

Neither NK1R antagonist affected the ***locomotor activity*** of NK1R-/- mice in the light zone, but both increased that of NK1R+/+ mice. It is noteworthy that this increase in the wild-type was evident at both 5 and 10 mg/kg RP 67580, whereas it was only evident at the higher drug dose for L 733060 (see below for comparisons between groups using the LSD Post-Hoc test).

RP 67580—5 mg/kg (see Fig 5.7 A)	$P = 0.039$
RP 67580—10 mg/kg (see Fig 5.7 A)	$P = 0.026$
L 733060—10 mg/kg (see Fig 5.7 B)	$P = 0.002$

- ***Other behaviours***

After pretreatment with an NK1R antagonist, the risk assessment behaviours (***FBA*** and ***SAP***) were not altered in NK1R-/- mice, but were reduced in NK1R+/+ mice to scores seen with the knockout (see below for the 'Genotype * Drug interactions):

FBA:	RP 67580 (see Fig 5.7 C)	$F_{2,18} = 34.9$	$P < 0.001$
	L 733060 (see Fig 5.7 D)	$F_{2,12} = 5.1$	$P < 0.05$
SAP:	RP 67580 (see Fig 5.7 E)	$F_{2,18} = 4.5$	$P < 0.05$
	L 733060 (see Fig 5.7 F)	NOT significant	

After ANCOVA, all these NK1R antagonist-induced genotype differences in the risk assessment behaviours were still observed ($F_{2,17} = 30.7$, $P < 0.001$; $F_{2,17} = 7.4$, $P < 0.01$ for RP 67580; $F_{2,11} = 4.5$, $P < 0.05$ for L 733060). This suggested that these genotype differences were not explained by a difference in ***locomotor activity*** in NK1R+/+ and NK1R-/- mice.

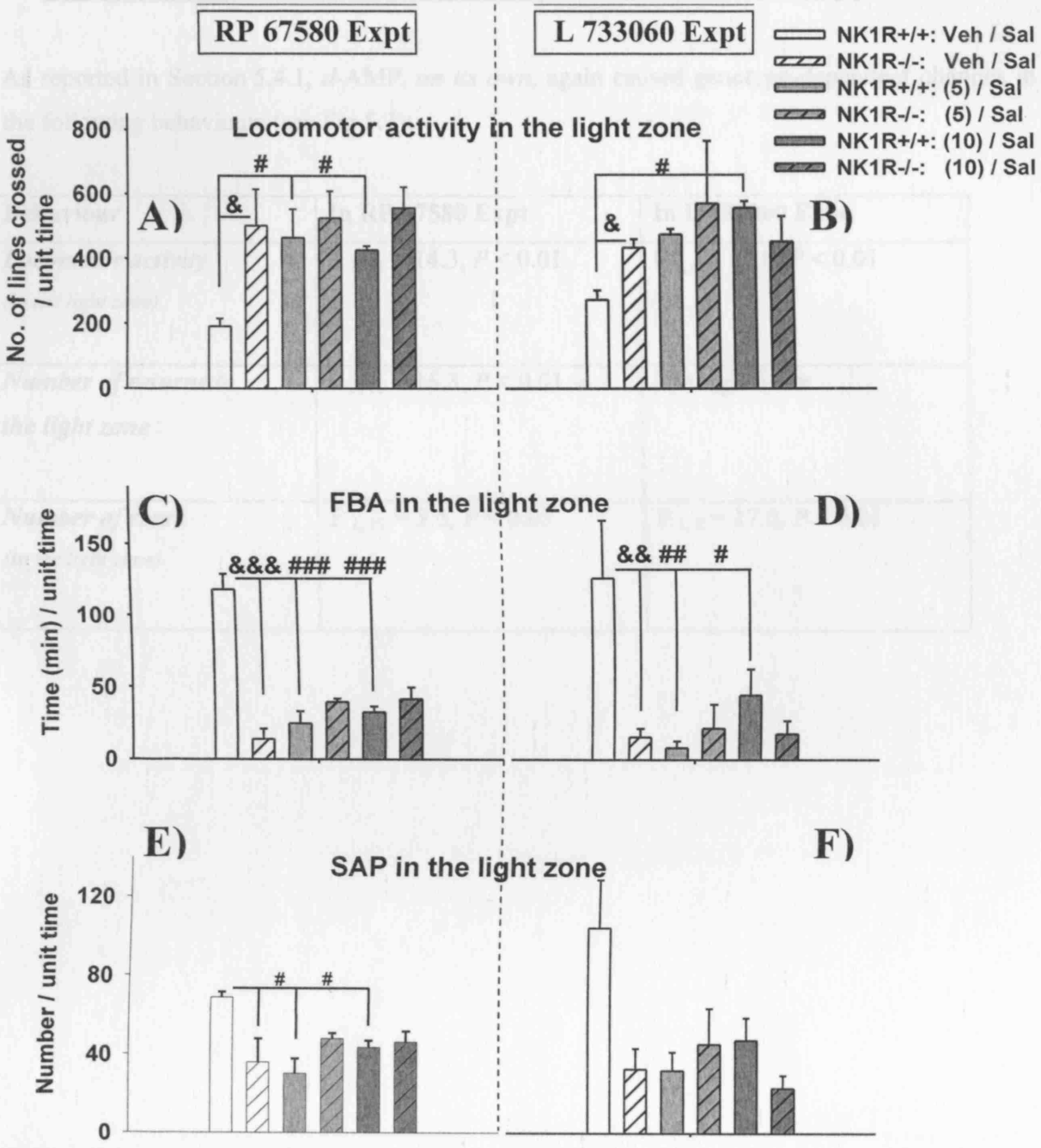


Fig 5.6 Behaviours demonstrating a 'Genotype*Drug' interaction in NK1R+/+ and NK1R-/- mice pretreated with the NK1R antagonist, RP 67580 (graphs A, C and E; N = 3~4/group) or L 733060 (graphs B, D and F; N = 3/group). Comparisons between individual groups were performed using the LSD Post-Hoc test, after obtaining significance for 'Genotype*Drug' interaction. Values are mean ± SEM. NK1R antagonist doses: (5)—5 mg/kg, (10)—10 mg/kg. Sal: saline. Veh: vehicle. FBA: Flat-Back Approach. SAP: Stretch-Attend Posture. NK1R+/+: # $P < 0.05$. ## $P < 0.01$. ### $P < 0.001$. NK1R+/+ *vs*. NK1R-/-: § $P < 0.05$. §§ $P < 0.01$. §§§ $P < 0.001$.

- **Effects of NK1R antagonism on the *d*-AMP-induced behavioural changes**

As reported in Section 5.4.1, *d*-AMP, *on its own*, again caused genotype-dependent changes in the following behaviours (see Fig 5.7):

Behaviour	**In RP 67580 Expt**	**In L 733060 Expt**
Locomotor activity *(in the light zone)*	$F_{1,11} = 14.3$, $P < 0.01$	$F_{1,8} = 17.0$, $P < 0.01$
Number of returns to the light zone	$F_{1,11} = 16.3$, $P < 0.01$	Not significant
Number of rears *(in the light zone)*	$F_{1,11} = 5.6$, $P < 0.05$	$F_{1,8} = 17.0$, $P < 0.01$

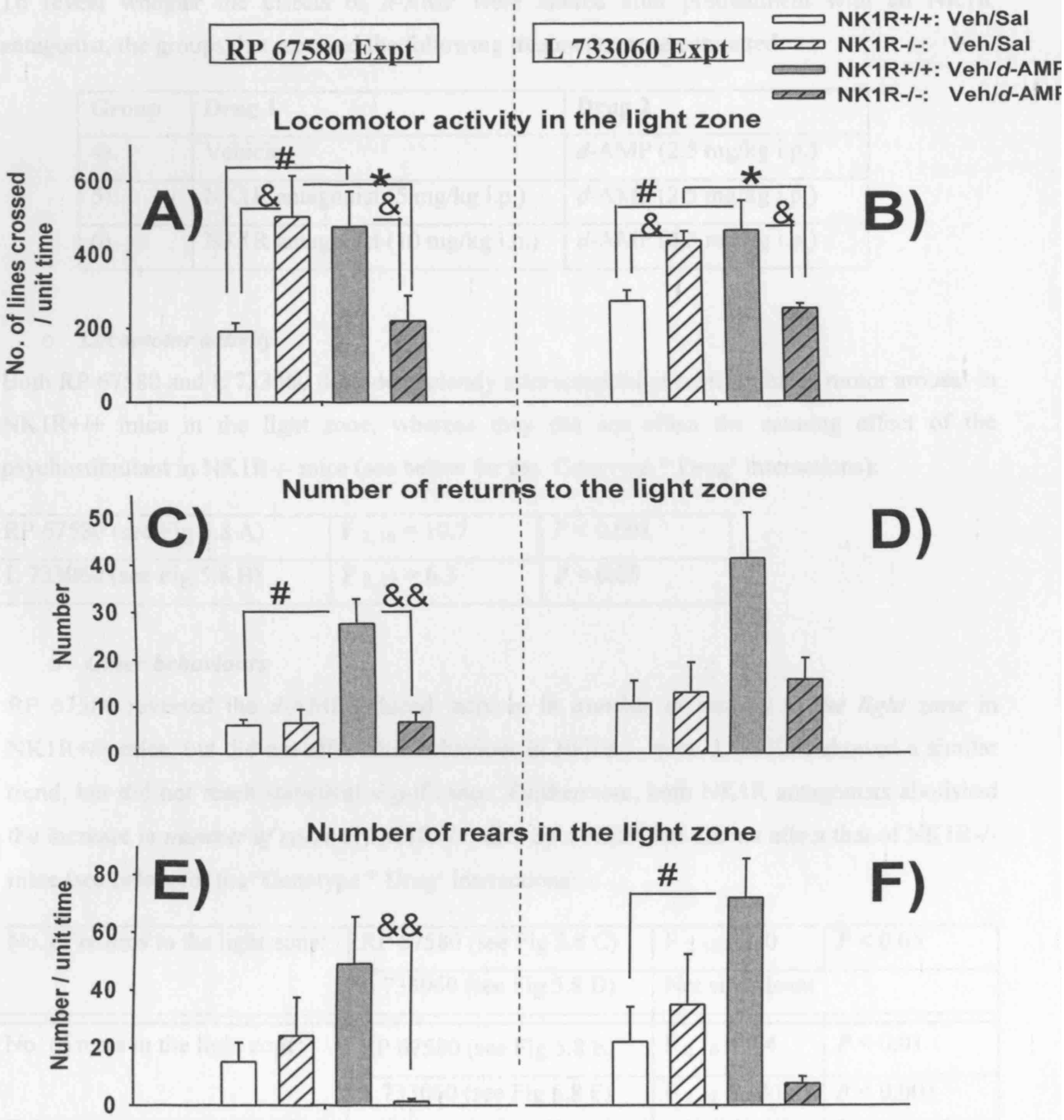


Fig 5.7 Behaviours demonstrating the genotype-dependent effects of *d*-AMP in the vehicle-pretreated NK1R+/+ and NK1R-/- mice. Mice used for the RP 67580 experiments: Fig A, C & E (N = 3~4/group); Mice used for the L 733060 experiments: Fig B, D & F (N = 3/group). Comparisons between individual groups were performed using the LSD Post-Hoc test, after obtaining significance for 'Genotype*Drug' interaction Values are mean ± SEM. Sal: saline; Veh: vehicle. NK1R+/+: # $P < 0.05$. NK1R-/-: * $P < 0.05$. NK1R+/+ *vs*. NK1R-/-: & $P < 0.05$. && $P < 0.01$.

To reveal whether the effects of *d*-AMP were altered after pretreatment with an NK1R antagonist, the groups that received the following treatments were compared:

Group	**Drug 1**	**Drug 2**
4).	Vehicle	*d*-AMP (2.5 mg/kg i.p.)
5).	NK1R antagonist (5 mg/kg i.p.)	*d*-AMP (2.5 mg/kg i.p.)
6)	NK1R antagonist (10 mg/kg i.p.)	*d*-AMP (2.5 mg/kg i.p.)

- ***Locomotor activity***

Both RP 67580 and L 733060 dose-dependently attenuated the *d*-AMP-induced motor arousal in NK1R+/+ mice in the light zone, whereas they did not affect the calming effect of the psychostimulant in NK1R-/- mice (see below for the 'Genotype * Drug' interactions):

RP 67580 (see Fig 5.8 A)	$F_{2,16} = 10.7$	$P < 0.001$
L 733060 (see Fig 5.8 B)	$F_{2,12} = 6.3$	$P < 0.05$

- ***Other behaviours***

RP 67580 reversed the *d*-AMP-induced increase in ***number of returns to the light zone*** in NK1R+/+ mice, but did not affect this behaviour in NK1R-/- mice. L 733060 showed a similar trend, but did not reach statistical significance. Furthermore, both NK1R antagonists abolished the increase in ***number of rears*** of NK1R+/+ mice by *d*-AMP, but did not affect that of NK1R-/- mice (see below for the 'Genotype * Drug' interactions):

No. of returns to the light zone:	RP 67580 (see Fig 5.8 C)	$F_{2,16} = 6.0$	$P < 0.05$
	L 733060 (see Fig 5.8 D)	Not significant	
No. of rears in the light zone:	RP 67580 (see Fig 5.8 E)	$F_{2,16} = 9.4$	$P < 0.01$
	L 733060 (see Fig 5.8 F)	$F_{2,12} = 14.0$	$P < 0.001$

After ANCOVA, there was no longer a genotype difference in ***number of returns to the light zone*** in RP 67580-pretreated animals. Hence, this apparent difference was solely due to the genotype difference in locomotor activity. However, the difference in ***number of rears*** was still evident ($F_{2,15} = 4.2$, $P < 0.05$ for RP 67580; $F_{2,11} = 5.7$, $P < 0.05$ for L 733060). This indicated that differences in locomotor activity did not account for this genotype difference.

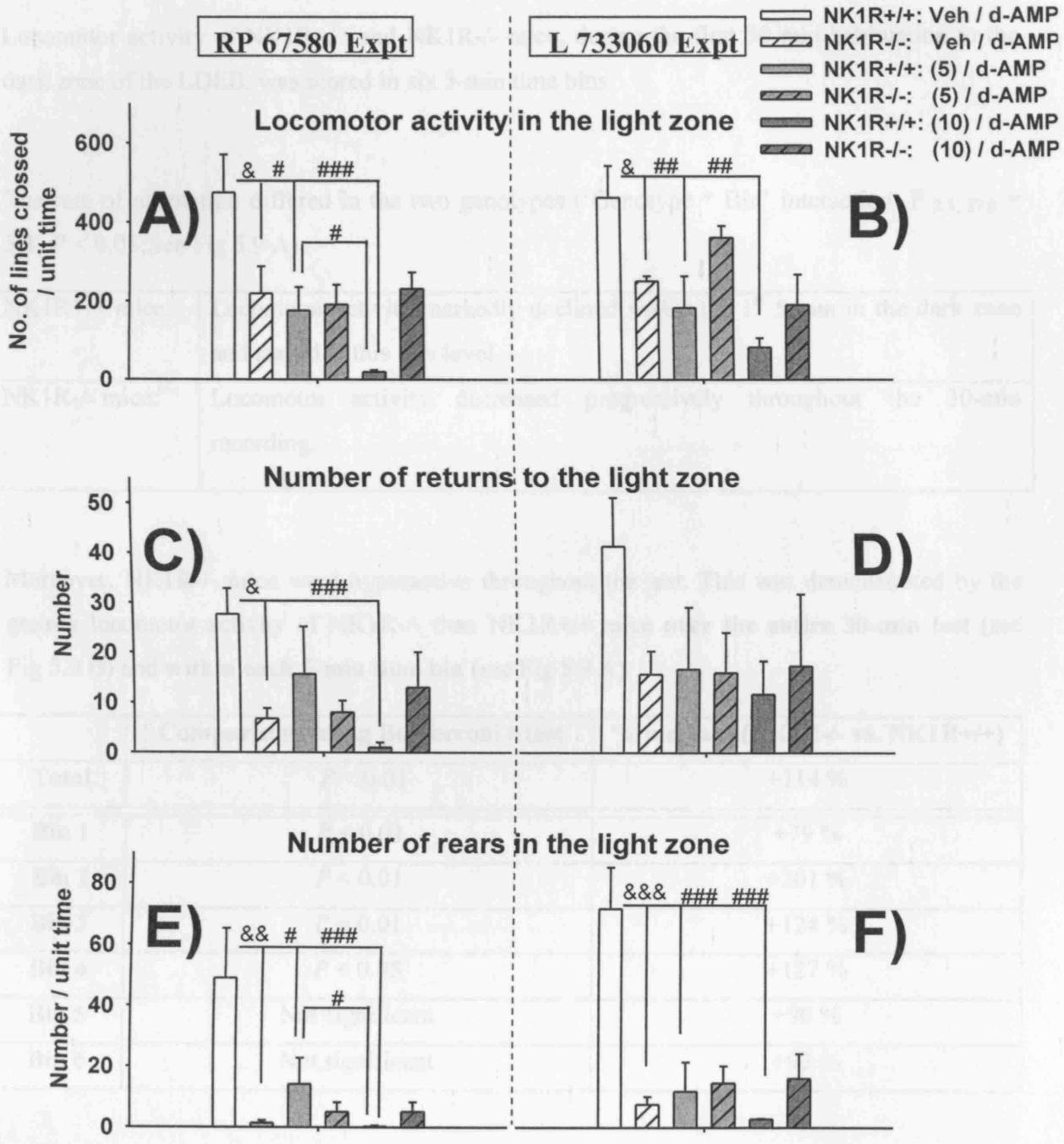


Fig 5.8 Pretreatment with RP 67580 (graphs A, C and E; N = 3~4/group) or L 733060 (graphs B, D and F; N = 3/group) abolished the genotype effects of *d*-AMP. Comparisons between individual groups were performed using the LSD Post-Hoc test, after obtaining significance for a 'Genotype*Drug' interaction Values are mean ± SEM. NK1R antagonist doses: (5)—5 mg/kg, (10)—10 mg/kg. Veh: vehicle. NK1R+/+: # $P < 0.05$. ## $P < 0.01$. ### $P < 0.001$. NK1R+/+ *vs*. NK1R-/-: & $P < 0.05$. && $P < 0.01$. &&& $P < 0.001$.

5.4.3. Locomotor activity during the 1st 30 min habituation to the dark zone

Locomotor activity of NK1R+/+ and NK1R-/- mice, during the first 30 min habituation to the dark zone of the LDEB, was scored in six 5-min time bins.

The rate of adaptation differed in the two genotypes ('Genotype * Bin' interaction: $F_{2.5, 27.9} = 3.1$, $P < 0.05$; see Fig 5.9 A):

NK1R+/+ mice:	Locomotor activity markedly declined *within* the 1st 5 min in the dark zone and stayed at this low level.
NK1R-/- mice:	Locomotor activity decreased progressively throughout the 30-min recording.

Moreover, NK1R-/- mice were hyperactive throughout the test. This was demonstrated by the greater locomotor activity of NK1R-/- than NK1R+/+ mice **over the entire 30-min test** (see Fig 5.9 B) and **within each 5-min time bin** (see Fig 5.9 A):

	Comparisons using Bonferroni t-test	**% increase (NK1R-/- vs. NK1R+/+)**
Total	$P < 0.01$	+114 %
Bin 1	$P < 0.01$	+79 %
Bin 2	$P < 0.01$	+201 %
Bin 3	$P = 0.01$	+124 %
Bin 4	$P < 0.05$	+127 %
Bin 5	Not significant	+90 %
Bin 6	Not significant	+93 %

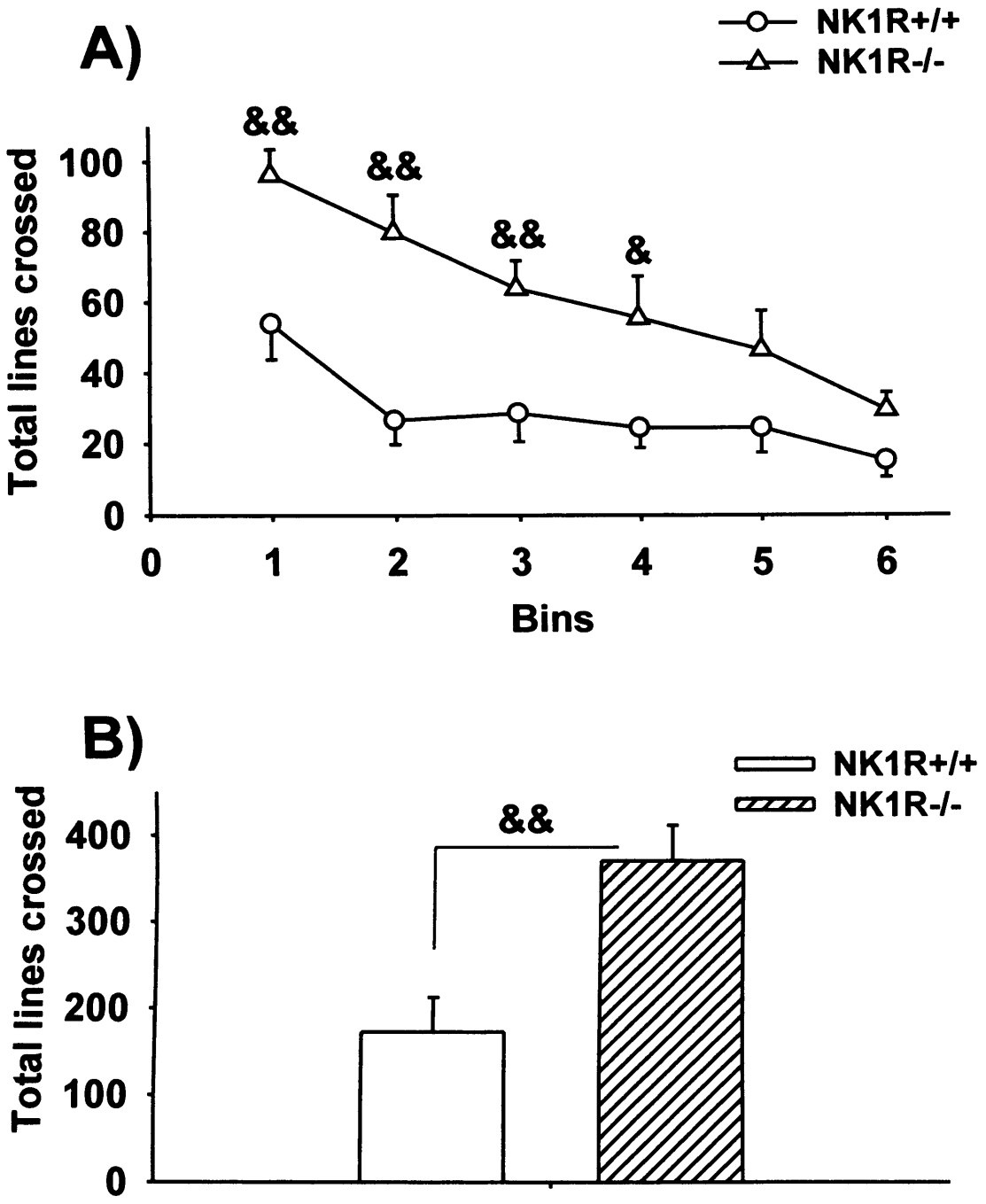


Fig 5.9 Locomotor activity of NK1R+/+ and NK1R-/- mice during the 1st 30 min habituation to the dark zone of the LDEB (N = 6~7/group). A) Locomotor activity within each 5-min time bin, and B) locomotor activity over the entire 30-min test. Genotype comparisons were performed using the Bonferroni t-test, after obtaining significance for a main effect of 'Genotype' or 'Bin', or their interaction. Values are mean ± SEM. NK1R+/+ *vs.* NK1R-/-: & $P < 0.05$. && $P < 0.01$.

5.5. Discussion

This study has revealed striking differences in the behavioural response of NK1R+/+ and NK1R-/- mice to the psychostimulants, *d*-AMP or MPH, in the LDEB. These genotype differences were abolished by pretreatment with an NK1R antagonist, RP 67580 or L 733060, which had no effects in NK1R-/- mice.

5.5.1. The effects of *d*-AMP and MPH that depended on genotype

As reported previously by our group (Herpfer *et al*, 2005; Fisher *et al*, 2007), NK1R-/- mice were again found to be hyperactive in the LDEB. Acute administration of *d*-AMP or MPH, while increasing the motor activity of NK1R+/+ mice, prevented the hyperactivity of the mutants. This is consistent with the finding that pretreatment with the NK1R antagonist, CP 99994 or LY 306740, blocks horizontal locomotion induced by i.p. injection of the psychostimulant, cocaine, in rats (Kraft *et al*, 2001).

The locomotor activation in NK1R+/+ mice by *d*-AMP or MPH is thought to be mediated by the drug-induced increase in central monoaminergic transmission. A large body of microdialysis evidence suggests that these psychostimulants cause locomotor activation by augmenting DA transmission in the mesolimbic system (Pijnenburg *et al*, 1976; Sharp *et al*, 1987; Kuczenski & Segal, 1989, 1991, 1997). Other studies revealed that NA also contributes to the expression of stimulant-induced behaviours (Kuczenski & Segal, 2001, 2002), perhaps *via* an interaction with DA systems (Darracq *et al*, 1998; Pascoli *et al*, 2001; Auclair *et al*, 2002; see Section 6.5.2). Moreover, serotonin systems in the brain might be involved as well (Segal, 1976; Jacobs & Fornal, 1997), although its role is still unclear, in part because drugs like MPH have little affinity for 5-HT transporters (Gatley *et al*, 1996).

In contrast to the drug effects in the wild-type, the hyperactivity of NK1R-/- mice was reduced by *d*-AMP. This indicated that NK1 receptors could be a crucial factor determining the locomotor stimulating effects of this drug. Nevertheless, how these receptors exert this effect remains unclear. Immunohistochemical studies revealed that NK1 receptors are densely located in the catecholamine-containing brain regions, such as the LC and the dorsal striatum (Shults *et al*, 1984), providing binding sites for their endogenous ligand, substance P, which is also present in high concentration in these brain areas (Ljungdahl *et al*, 1978; Shults *et al*, 1984; Pammer *et*

al, 1990; Chen *et al*, 2000). Substance P is known to excite the LC *via* NK1 receptors (Shen *et al*, 1992), hence increasing NA release at the terminal sites in the **PFC**. There is microdialysis evidence that NK1 receptors are needed to maintain the PFC catecholaminergic response to stress, as blocking NK1 receptors with a selective NK1R antagonist, GR 205171, attenuated stress-induced rise of extracellular NA and DA in the rat and gerbil medial PFC (Renoldi & Invernizzi, 2006). Moreover, slice studies found that substance P increases DA release in the **dorsal striatum**, possibly by targeting NK1 receptors on striatal acetylcholine-producing interneurones (Gerfen, 1991; Khan *et al*, 2000; Krolewski *et al*, 2005). Since both the PFC and the striatal catecholamines are implicated in the locomotor arousing effects of *d*-AMP (Darracq *et al*, 1998; Gonzalez-Nicolini & McGinty, 2002; also see Section 1.2.3 and Section 6.1), it is possible that disruption of NK1 receptors somehow modifies transmission of NA and / or DA in cortico-striatal circuits in NK1R-/- mice, which resulted in the suppression of their hyperactivity by *d*-AMP. This hypothesis was tested in Chapter 6 (for DA) and 7 (for NA).

The calming effects of *d*-AMP in NK1R-/- mice echoed the therapeutic effects of this drug in the treatment of ADHD (see Wilens *et al*, 2002), and suggested the possibility of NK1R-/- mice as a novel rodent model of this disorder. To further test this hypothesis, the behaviour of NK1R-/- mice was compared with that of NK1R+/+ controls in response to acute administration of MPH, another first-line medication used to treat ADHD.

The results showed that MPH, like *d*-AMP, had genotype-dependent effects on the animals' motor activity: line-crossing of NK1R+/+ mice was markedly increased by MPH, whereas that of NK1R-/- mice did not differ from the wild-type controls after the drug treatment, although it did not differ from the knockout controls, either. These findings imply that MPH did have an inhibitory effect on the motor response of the mutants, but this effect was not as large / consistent as that of *d*-AMP. In fact, MPH does not reduce the hyperactivity of the Spontaneous Hypertensive Rat (SHR: the best validated rat model of ADHD), either: rather it increased the motor response of these rats in, for example, the open-field test (Yang *et al*, 2006).

There is evidence that symptoms of ADHD are relieved by only relatively low, but not high, doses of psychostimulants (Kuczenski & Segal, 2002, 2005). Therefore, the finding that MPH did not significantly prevent the hyperactivity of NK1R-/- mice could be due to the drug dose used in the current study (2.5 mg/kg) being inappropriate. Nevertheless, this has been found

unlikely to be the case, as a pilot experiment showed that MPH, at 1.5 ~ 5 mg/kg (i.p.), reduced hyperactivity of NK1R-/- mice (see Appendix II). Further studies will be needed to reveal why locomotor activity of the mutants after MPH did not differ from that of the drug-free mutants.

Moreover, using a similar protocol, colleagues in this lab showed that the tricyclic antidepressant, desipramine, reduced locomotor activity of NK1R-/-, but not NK1R+/+, mice (Herpfer *et al*, 2005). Tricyclic antidepressants, like desipramine, are effective in improving cognitive impairments associated with ADHD, and are particularly useful in stimulant failures or when anxiety / depressive symptoms co-occur with ADHD (Spencer *et al*, 1998). Therefore, reversal of the hyperactivity of the mutants by desipramine strengthens the predictive validity of NK1R-/- mice as a screen for ADHD drugs. Nevertheless, it is noteworthy that desipramine has α_1-adrenoceptor antagonist properties (Subhash *et al*, 2003), and these adrenoceptors in the PFC are thought to play a role in motor activity (Darracq *et al*, 1998). Therefore, we cannot rule out the possibility that the reduction of the mutants' hyperactivity by desipramine is mediated by α_1-adrenoceptors, only. Moreover, other effects of desipramine, such as histamine (H1)-receptor antagonist properties (see Rang *et al*, 2003) which lead to sedation, might also contribute the different effects of this drug in the two genotypes.

Other behaviours monitored in the LDEB, e.g. ***number of returns to the light zone*** and ***number of rears***, also indicate the state of arousal of animals. These behaviours did not differ in the drug-free NK1R+/+ and NK1R-/- mice. Nevertheless, administration of *d*-AMP or MPH markedly increased ***number of returns to the light zone*** in NK1R+/+ mice, but did not affect that in NK1R-/- mice. MPH also caused a genotype-dependent increase in ***number of rears*** in NK1R+/+ mice, only. Taken together, these data lend further support to the arousing effects of psychostimulants in NK1R+/+, but not NK1R-/-, mice.

5.5.2 The effects of NK1R antagonism on the mouse behaviour in the LDEB

The above findings have revealed abnormal motor responses of NK1R-/- mice to psychostimulants. However, it is important to ascertain whether this abnormality rests on a lack of functional NK1 receptors, or any developmental / compensatory changes (e.g. up- or downregulation of other gene products). For this reason, experiments were also performed to test

the effects of pretreatment with a selective NK1R antagonist, either RP 67580 or L 733060, on the *d*-AMP-induced behavioural changes in NK1R+/+ and NK1R-/- mice.

- **Effects of NK1R antagonists alone** ***(i.e. in mice which did not receive d-AMP)***

The acute administration of the NK1R antagonist, RP 67580 or L 733060, turned NK1R+/+ into 'pseudo-NK1R-/-' mice, which behaviourally resembled NK1R-/- mice:

NK1R+/+ mice that were pretreated with the NK1R antagonist became as ***hyperactive*** as NK1R-/- mice in the light zone of the LDEB. Interestingly, while RP 67580 augmented the locomotor response of NK1R+/+ mice at both 5 and 10 mg/kg, L 733060 only exert this effect at the higher dose. This is probably because L 733060 has lower affinity than RP 67580 for the rat / mouse NK1 receptor (Harrison *et al*, 1994; Kramer *et al*, 1998). Alternatively, it could be due to a lower bioavailability and / or less absorption of L 733060 than RP 67580.

In contrast, the hyperactivity of NK1R-/- mice was not affected by either NK1R antagonist at either dose. This confirmed the selectivity of the two NK1R antagonists, and suggested that the hyperactivity of the mutants was caused by functional disruption of their NK1 receptors.

This conclusion is further supported by the genotype-dependent changes in the risk assessment behaviour. After pretreatment with RP 67580 or L 733060, ***FBA*** and ***SAP*** of NK1R+/+ mice were decreased to the knockout level, whereas these measures of NK1R-/- mice were not altered.

- **Effects of NK1R antagonism on the *d*-AMP-induced behavioural changes**

Pretreatment with an NK1R antagonist abolished the genotype differences in response to *d*-AMP:

In NK1R+/+ mice, RP 67580 and L 733060 dose-dependently diminished the *d*-AMP-induced increase in ***locomotor activity*** as well as other behaviours that indicate arousal (e.g. ***number of returns to the light zone*** and ***number of rears***).

In comparison, the calming effect of the psychostimulant in NK1R-/- mice was not altered by either NK1R antagonist. This again confirmed that the NK1R antagonists are selective for the mouse NK1 receptor. It also indicated that the prevention of the motor arousal of NK1R-/- mice by *d*-AMP rests on a lack of functional NK1 receptors.

5.5.3. Locomotor activity during the 1st 30 min habituation to the dark zone

Finally, NK1R-/- mice had greater locomotor activity than NK1R+/+ mice, during the 1st 30 min habituation to the dark zone of the LDEB. This is again consistent with our previous finding that the mutants are hyperactive in the LDEB (Herpfer *et al*, 2005; Fisher *et al*, 2007). This genotype difference in locomotor activity proved that the dark zone, although of low light intensity, was perceived as novel and possibly stressful to NK1R+/+ and NK1R-/- mice when the animals were first placed in this compartment. Hence, the lack of a genotype difference in locomotor activity in the dark zone, reported in Section 5.4.1, is not because that this compartment was not novel enough to elicit a genotype difference. Instead, it is because of the 90-min habituation prior to the tests.

Moreover, during the 1st 30 min in the dark zone, NK1R-/- mice habituated more slowly than NK1R+/+ mice. This is in keeping with the impaired habituation to novelty seen in several animal models of ADHD, such as the DAT knock-out mouse (Gainetdinov *et al*, 1999) or knock-down mouse (Zhuang *et al*, 2001), and the thyroid receptor β PV knock-in mouse (Siesser *et al*, 2005). This impairment could be attributed to over-expression and / or increased functioning of DA D4 receptors (a candidate gene in ADHD), as these receptors are associated with novelty seeking (Benjamin *et al*, 1996; Ebstein *et al*, 1997), and are though to be important for expression of hyperactivity and disrupted behavioural inhibition in ADHD (Avale *et al*, 2004).

However, to our knowledge, there are no clinical reports indicating impaired habituation to novel environments in patients with ADHD. Rather, Sleator & Ullmann (1981) revealed that about 80% ADHD patients show no sign of hyperactivity during office diagnosis, but only show disrupted behaviour at home / school, which is assumed to be a familiar environment.

5.6. Summary

- **The effects of *d*-AMP and MPH are genotype-dependent:**

  –NK1R+/+: ↑ *locomotor activity*

  ↑ behaviours indicative of arousal (*no. of returns to the light zone & rearing*)

  – NK1R-/-: ↓ *locomotor activity*

  No change in *no. of returns to the light zone* or *rearing*

  Hence, *d*-AMP needs NK1R to express the locomotor-activating effects.

- **Pretreatment with an NK1R antagonist turned NK1R+/+ into NK1R-/- mice, but had no effects in the mutants:**

  Hence: --the NK1R antagonists are selective for the mouse NK1 receptors.

  --the hyperactivity of NK1R-/- mice and its prevention by *d*-AMP were due to disruption of functional NK1 receptors.

- **During the 1st 30-min habituation to the dark zone of the LDEB:**

  –Locomotor activity of NK1R-/- mice was greater than that of NK1R+/+ mice.

  Thus suggesting the absence of overall hyperactivity of NK1R-/- mice in the dark zone after the 90-min habituation, is because animals have habituated to this compartment.

  –NK1R-/- mice habituated more slowly than NK1R+/+ mice

  Indicating impaired habituation to novelty in NK1R-/- mice, as in other ADHD models (e.g. DAT knock-out or knock-down mouse, and the thyroid receptor β PV knock-in mouse).

So far, Chapter 4 and 5 have reported abnormal *PFC catecholamine response* and *behavioural response* to *d*-AMP and MPH (the two first-line medications for ADHD) in NK1R-/- mice, respectively. Thus the findings point to the relevance of these mutants to ADHD. In the next two chapters, the validity of NK1R-/- mice as a rodent model of this disorder was further tested, by characterizing their DA and NA transmission in cortico-striatal circuits, following systemic administration of *d*-AMP in the LDEB.

Chapter 6. Abnormal dopaminergic response in the dorsal striatum and the prefrontal cortex of NK1R-/- mice following acute administration of d-amphetamine in the light / dark exploration box

6.1. Introduction

The hyperactivity of NK1R-/- mice and their abnormal behavioural response to *d*-AMP and MPH, reported in Chapter 5, pointed to the possibility of NK1R-/- mice as a model of ADHD. To date, the etiology of ADHD is still not clear. However, by using various techniques (e.g. microdialysis in animals; neuroimagining and genetic studies in animals and humans), there is a growing body of evidence that this disorder is associated with impaired DA transmission in the PFC and the dorsal striatum as well as their associated pathways, i.e. cortico-striatal circuits. This is because DA transmission in these brain regions is implicated in cognition and motor activity, and is targeted by drugs that are used to treat ADHD (e.g. see Arsten & Li, 2005; Madras *et al*, 2005; Sagvolden *et al*, 2005).

In the experiments described in this chapter, DA efflux in the dorsal striatum was compared in NK1R+/+ and NK1R-/- mice. This brain region was chosen because DA released from the nigrostriatal terminals has a major role in initiation and execution of movement, as evident by Parkinson's disease (Ehringer & Horynkiewicz, 1960; Birkmayer & Hornykiewicz, 1961, see Iverson & Iverson, 2007). Mediation of motor activity by striatal DA is thought to occur, at least in part, through stimulation of striatal D1 receptor-expressing medium spiny neurones. This is based on the evidence that intrastriatal administration of selective D1 receptor agonists increases motor activity in rats (Bishop & Walker, 2003; Krolewski *et al*, 2005). This process has been hypothesized to occur *via* the following mechanism:

The striatal medium spiny neurones, which express D1 receptors, project directly to the substantia nigra (SN). At the striatonigral terminals, GABA is released, which plays a crucial role in initiation of movements (Kilpatrick *et al*, 1982; see Alexander & Crutcher, 1990). This function is also thought to be mediated by substance P, which is co-expressed with GABA in the striatonigral neurones (Gerfen & Young III, 1988; Lee *et al*, 1997). This is based on the evidence that intranigral administration of this peptide elicits motor activity in rats (James & Starr, 1977). The action of substance P is exerted *via* stimulation of their preferred NK1 receptors, which are localized in the SN (Futami *et al*, 1998). The role of these nigral NK1 receptors in movement is supported by a recent behaviour test, which showed that bilateral intranigral infusion of the

selective NK1R antagonist, LY 306740, dose-dependently reduced the locomotor response to intrastriatal administration of the D1 receptor agonist in rats (Bishop & Walker, 2004). Moreover, activation of the nigral NK1 receptors can enhance release of DA from the nigrostrital terminals in the dorsal striatum, as evident by push-pull cannulae and *in vivo* microdialysis studies (Baruch *et al*, 1988; Reid *et al*, 1990). This positive feed-forward mechanism might help sustain continued movements (see Fig 6.1 A).

In addition to the striatonigral projections, the striatal D1-expressing neurones that co-release GABA and substance P also project collaterally onto acetylcholine (ACh) interneurones in the dorsal striatum (Lee *et al*, 1997; Li *et al*, 2000), where striatal NK1 receptors are localized (Gerfen, 1991; Kaneko *et al*, 1993). Using whole-cell patch clamping, there is evidence that administration of substance P directly depolarizes striatal ACh interneurones (Aosaki & Kawaguchi, 1996), *via* activation of NK1 receptors located on ACh neurones (Quartara & Maggi, 1997). These striatal NK1 receptors have also been found to be integral to motor activity. In a recent open-field test, Krolewski *et al* (2005) observed that bilateral intrastriatal infusion of the NK1R antagonist, LY 306740, reduced the locomotor activation caused by the D1 receptor agonist which was also administered locally into the striatum. Therefore, these authors suggested that NK1 receptors might have a permissive role in the behavioural responses initiated by striatal D1 receptor stimulation. This process could be mediated, in part, through interactions with striatal ACh interneurones, as both microdialysis and slice studies reported that selective striatal NK1R agonism augments ACh release in the dorsal striatum (Arenas *et al*, 1991; Steinberg *et al*, 1995; Preston *et al*, 2000; Kemel *et al*, 2002), while NK1R antagonism inhibits the local release of ACh caused by striatal D1 receptor stimulation (Anderson *et al*, 1994; Acquas & Di Chiara, 1999) (see Fig 6.1 B).

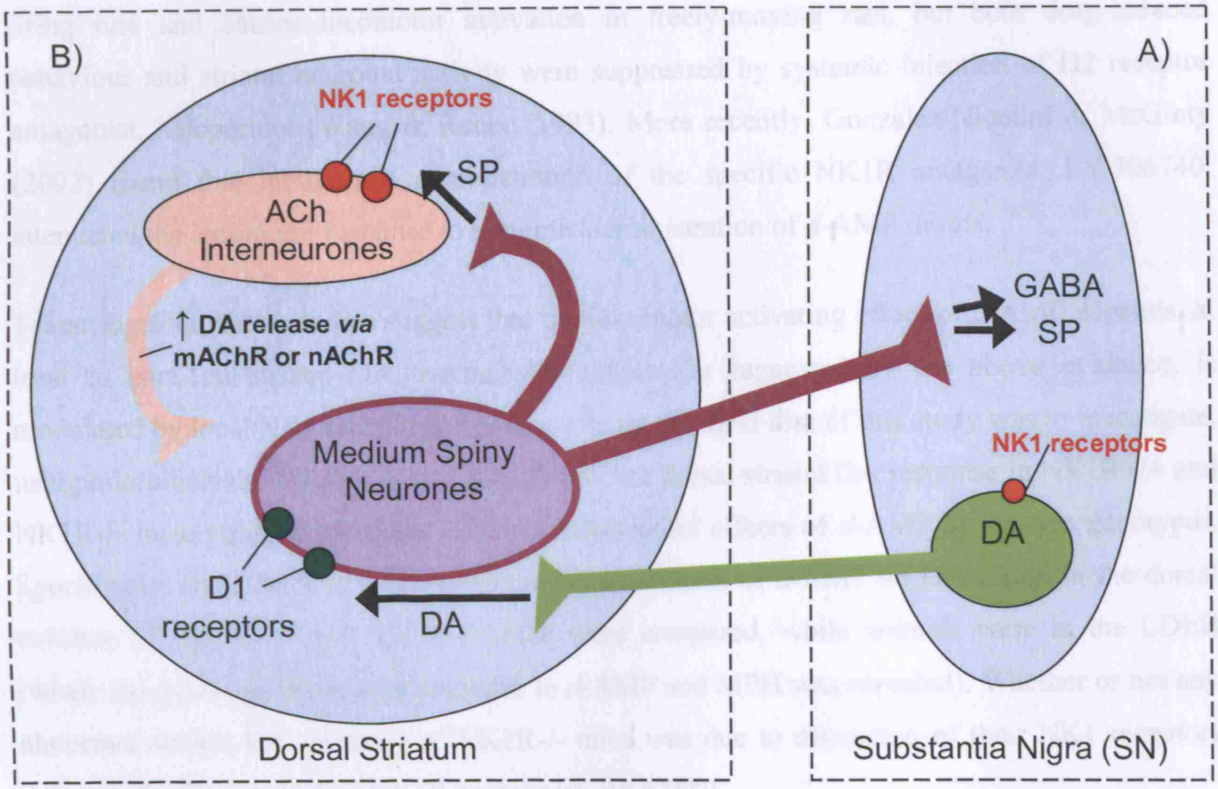


Fig 6.1 Diagrammatic illustration of mechanisms which have been hypothesized to underlie the striatal DA modulation of motor behaviour. A) The striatonigral D1 receptor-expressing neurones projecting to the substantia nigra, where GABA and substance P (SP) are co-released; B) The striatonigral D1 receptor-expressing neurones projecting axon collaterals onto acetylcholine (ACh) interneurones, where striatal NK1 receptors are localized. mAChR and nAChR: muscarinic and nicotinic acetylcholine receptors, respectively.

Over many years, the hyperlocomotor response to psychostimulants (e.g. *d*-AMP) has been thought to result mostly from the drug-induced increase in DA release in the NAc, whereas DA in the dorsal striatum is thought to account mainly for stereotypy elicited by the drugs (Kelly *et al*, 1975; Wise, 1996). However, in the current project, the dorsal striatal DA response to *d*-AMP in NK1R+/+ and NK1R-/- mice was studied. This is because there is evidence that both DA transmission and NK1 receptor activation in this subcortical structure modulate the motor stimulating effect of *d*-AMP. For instance, after lesioning the rat dorsal striatum with the neural toxin, 6-OHDA, the *d*-AMP-induced locomotor activation is no longer apparent (Antoniou *et al*,

1998). There is also evidence that intrastriatal infusion of *d*-AMP increases striatal neuronal firing rate and causes locomotor activation in freely-moving rats, but both drug-induced behaviour and striatal neuronal activity were suppressed by systemic injection of D2 receptor antagonist, haloperidol (Wang & Rebec, 1993). More recently, Gonzalez-Nicolini & McGinty (2002) found that intrastriatal administration of the specific NK1R antagonist, LY 306740, attenuated the locomotor response to systemic administration of *d*-AMP in rats.

Taken together, these studies suggest that the locomotor activating effect of *d*-AMP depends, at least in part, on striatal DA transmission which, as suggested by the above evidence, is modulated by local NK1 receptors. For this reason, this **first aim** of this study was to investigate, using microdialysis, whether any differences in the dorsal striatal DA response in NK1R+/+ and NK1R-/- mice could explain the different behavioural effects of *d*-AMP in the two genotypes. Specifically, the effects of acute systemic administration of *d*-AMP on DA efflux in the dorsal striatum of NK1R+/+ and NK1R-/- mice were compared, while animals were in the LDEB (where the genotype-dependent response to *d*-AMP and MPH was revealed). Whether or not any abnormal striatal DA response of NK1R-/- mice was due to disruption of their NK1 receptors was tested using a selective NK1R antagonist, RP 67580.

The PFC DA, which is released from mesocortical DA neurones projected from the VTA, also influences the behavioural response to psychostimulants. This is believed to occur *via* the inhibitory modulation of DA activity in the subcortical areas by that in the mesocortical system, as first revealed by Carter and Pycock in 1980. These authors showed that lesioning the rat PFC DA systems with 6-OHDA, while leaving PFC NA systems relatively unaffected by using desipramine, increased DA turnover (the DOPAC / DA ratio) in the dorsal striatum and the NAc. The cortical lesions also enhanced spontaneous motor activity and amphetamine-induced stereotyped behaviour. The coupling between DA transmission in the cortical and subcortical areas has been suggested to be mediated by the inhibition of the PFC glutamatergic projection to the subcortical areas (see Fig 1.3 in Section 1.2.3.2).

Due to this anatomical linkage between the dorsal striatum and the PFC, this study also investigated (the **second aim)** whether the atypical motor response of NK1R-/- mice to *d*-AMP could be due to their abnormal PFC DA response to the drug. Again, the NK1R antagonist,

RP 67580, was used to test whether or not the abnormal cortical DA response of the mutant (if any) could be a direct result of disruption of their NK1 receptors.

Finally, neuroimaging studies, using SPECT, have revealed that individuals with ADHD have increased ligand binding at DAT in the dorsal striatum, which indicates lower DA transmission in this brain region (Dougherty *et al*, 1999; Krause *et al*, 2000). Moreover, MRI studies showed a smaller size of the caudate nucleus in patients with ADHD, but the reports on the lateralization of this abnormality are inconsistent. While Hynd *et al* (1993) and Fillipek *et al* (1997) found a loss of the normal 'greater left than right' asymmetry in the caudate nucleus in ADHD, as a result of reduced left caudate volume, Castellanos *et al* (1994, 1996, 2001, 2002) and Mataró *et al* (1997) reported the opposite. Therefore, the **third aim** of the experiments describe in this chapter was to compare, in tissue homogenates, DA and protein concentrations in both the left and right dorsal striatum of NK1R+/+ and NK1R-/- mice.

6.2. Aim:

- Compare basal DA efflux in the **dorsal striatum** and the **PFC** of NK1R+/+ and NK1R-/- mice, as well as their striatal and cortical DA response to acute administration of *d*-AMP.
- Investigate whether any abnormalities of DA transmission in NK1R-/- mice could be due to a lack of their functional NK1 receptors (by using the NK1R antagonist, RP 67580)

} **Using *in vivo* microdialysis**

- Compare DA concentration in the striatal homogenates of NK1R+/+ and NK1R-/- mice using HPLC, and determine striatal protein concentration of the two genotypes using protein assays.

6.3. Protocols

6.3.1. DA efflux in the dorsal striatum of NK1R+/+ and NK1R-/- mice in the LDEB

A microdialysis probe was inserted into the dorsal striatum (from bregma; in mm: AP +1.10, ML +1.5, DV -3.3) under halothane anaesthesia, as described in Section 2.3.5.2. On the day after surgery, microdialysis experiments were carried out, as described in Section 2.3.5.3. NK1R+/+ and NK1R-/- mice were randomly assigned for one of the 4 treatments (The following doses were chosen as they were found effective in Chapter 5):

Group	Drug 1	Drug 2
1).	Vehicle	Saline
2).	RP 67580 (5 mg/kg i.p.)	Saline
4)	Vehicle	*d*-AMP (2.5 mg/kg i.p.)
5)	RP 67580 (5 mg/kg i.p.)	*d*-AMP (2.5 mg/kg i.p.)

Microdialysis was performed between 12:00 h and 15:40 h. Ringer's solution was perfused down the probe throughout the experiment, and DA dialysate samples were collected at 20 min intervals. First, basal DA efflux was monitored while animals were in the home cage. After at least three consecutive stable basal DA samples had been obtained, mice were placed in the dark zone of the LDEB for a 90-min habituation, during which time the treatments were given. After the habituation, mice were transferred to the light zone and confined in this compartment, which allowed measurement of DA efflux in the environment where hyperactivity of NK1R-/- mice is most apparent (see Fig 6.2).

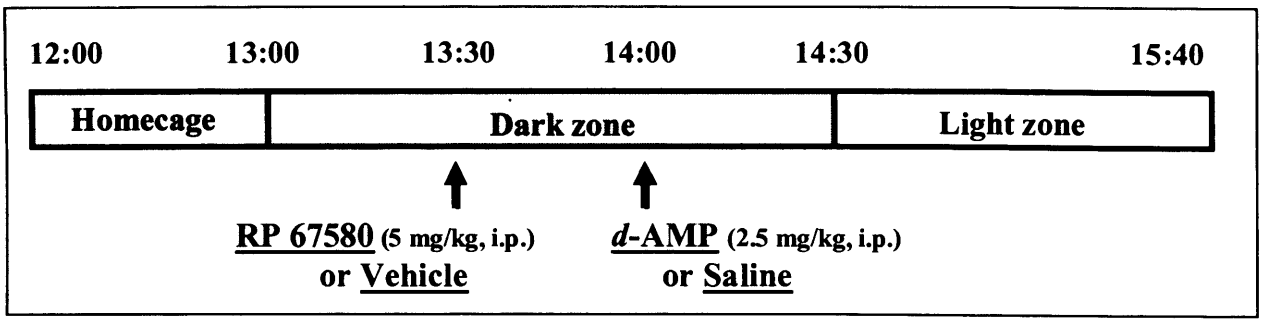


Fig 6.2 Protocol for the *in vivo* microdialysis experiment in the LDEB.

6.3.2. DA efflux in the PFC of NK1R+/+ and NK1R-/- mice in the LDEB

The microdialysis probe was inserted into the PFC (from bregma; in mm: AP +2.10, ML +1.0, DV -2.0) under halothane anaesthesia. On the day of microdialysis, the same protocol was used as described in Section 6.3.1.

6.3.3. Striatal DA & protein concentrations in the mouse striatal homogenates

• *Dissection*

Mice were killed by a Schedule 1 procedure (blow to the head and cervical dislocation). Brain was removed, the striatum was dissected on ice and stored at -20°C until use.

• *Homogenization*

Striatal tissue was thawed and weighed, and then homogenized in 100 µl 0.1 M $HCLO_4$ per 2 mg tissue. After 20 strokes of the homogenizer, the homogenate was transferred to a centrifuge tube. The homogenizing tube was further washed four times in 100 µl 0.1 M $HCLO_4$ and this solution was added to the centrifuge tube.

• *Centrifugation*

The homogenate was centrifuged at 13,000 x g for 5 min.

• *Measurement of DA concentrations*

The supernatant was diluted 1:25 with Ringer's solution, and 50 µl was injected into the HPLC.

• *Estimate of protein contents*

The protein pellet was first denatured in 1 ml of 0.5 M NaOH overnight. The next day, this protein solution was diluted 1:2 with 0.5 M NaOH. The striatal protein contents were determined using a Bicinchoninic Acid (BCA) kit (Sigma, Missouri). 0.1 ml diluted sample was added to 2 ml BCA Working Reagent. The mixture was incubated at 60°C for 20 min, followed by cooling to room temperature. Finally, the absorbance of the solution was measured at 630 nm using a photometer (D.A. Pitman Ltd, Surrey). The protein content of each sample was estimated from a standard curve, using bovine serum albumin (BSA; Sigma, Missouri).

6.4. Results

6.4.1. DA efflux in the dorsal striatum of NK1R+/+ and NK1R-/- mice in the LDEB

A 5-way ANOVA was carried out across all 8 groups. 'Genotype', 'RP 67580 (Drug 1)' and '*d*-AMP (Drug 2)' were considered as between-subjects factors. 'Bin' and 'Time' [T-40~0 (basals) *vs.* T80~120 (DA efflux after treatment with *d*-AMP)] were regarded as within-subjects factors.

The results showed an interaction of 'Genotype * RP 67580* *d*-AMP * Bin' ($F_{1,25} = 10.5$, $P < 0.01$). This indicated that DA efflux in the dorsal striatum depended on *genotype*, *RP 67580 pretreatment* and *d-AMP*.

The data reported in this section come from the same fully randomized study, but are discussed in two sub-sections (see Fig 6.3 ~ 6.5) for clarity.

- ***The effects of NK1R antagonism, on basal DA efflux in the dorsal striatum***

In the dorsal striatum, there was no difference in basal DA efflux in NK1R+/+ (24.5 ± 0.9 fmol / 20min) and NK1R-/- mice (25.7 ± 0.8 fmol / 20min). Further, the pretreatment with the NK1R antagonist, RP 67580, did not alter the striatal DA response in either genotype (see Fig 6.3).

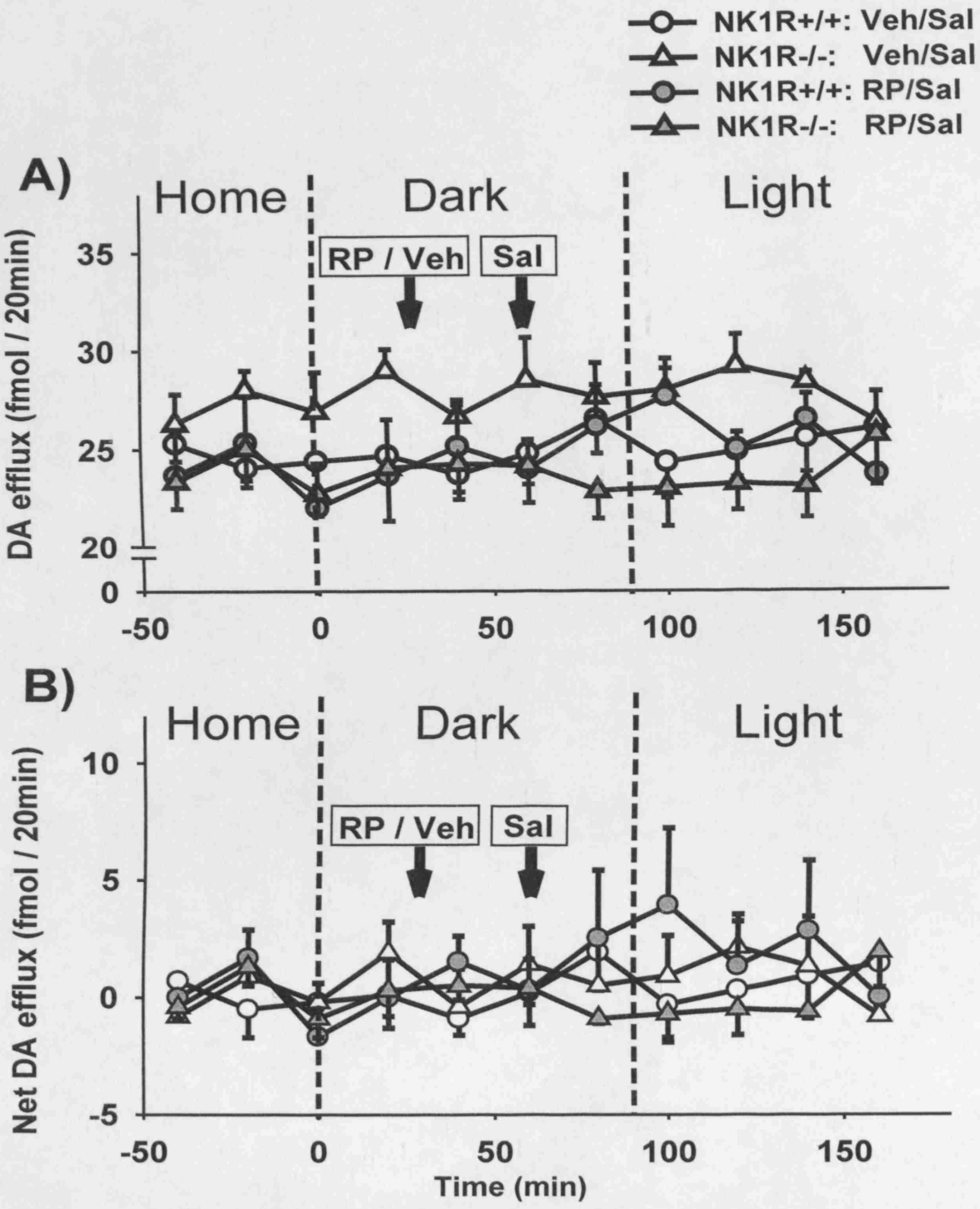


Fig 6.3 A) Raw data and B) the net changes showing basal DA efflux in the dorsal striatum of NK1R+/+ and NK1R-/- mice in the LDEB, and how this was affected by the NK1R antagonist, RP 67580 (N = 3~4/group). RP: PR 67580; Sal: saline; Veh: vehicle.

- ***The effects of NK1R antagonism on the striatal DA response to d-AMP***

To investigate the effect of *d*-AMP, alone, on striatal DA efflux, the NK1R+/+ and NK1R-/- mice that received treatments of 'vehicle / saline' and 'vehicle / *d*-AMP' were compared. The data revealed that the striatal DA response to *d*-AMP differed in the two genotypes:

'Genotype * *d*-AMP * Bin' interaction:	$F_{1,13} = 7.0$	$P < 0.05$
'Genotype * *d*-AMP' interaction:	$F_{1,13} = 7.0$	$P < 0.05$

Specifically, *d*-AMP increased striatal DA efflux in NK1R+/+ mice (*cf.* Basals: $F_{1,3} = 14.7$, $P < 0.05$; *cf.* NK1R+/+ (Veh/Sal): $F_{1,9} = 16.2$, $P < 0.01$), but did not affect that of NK1R-/- mice (see Fig 6.4).

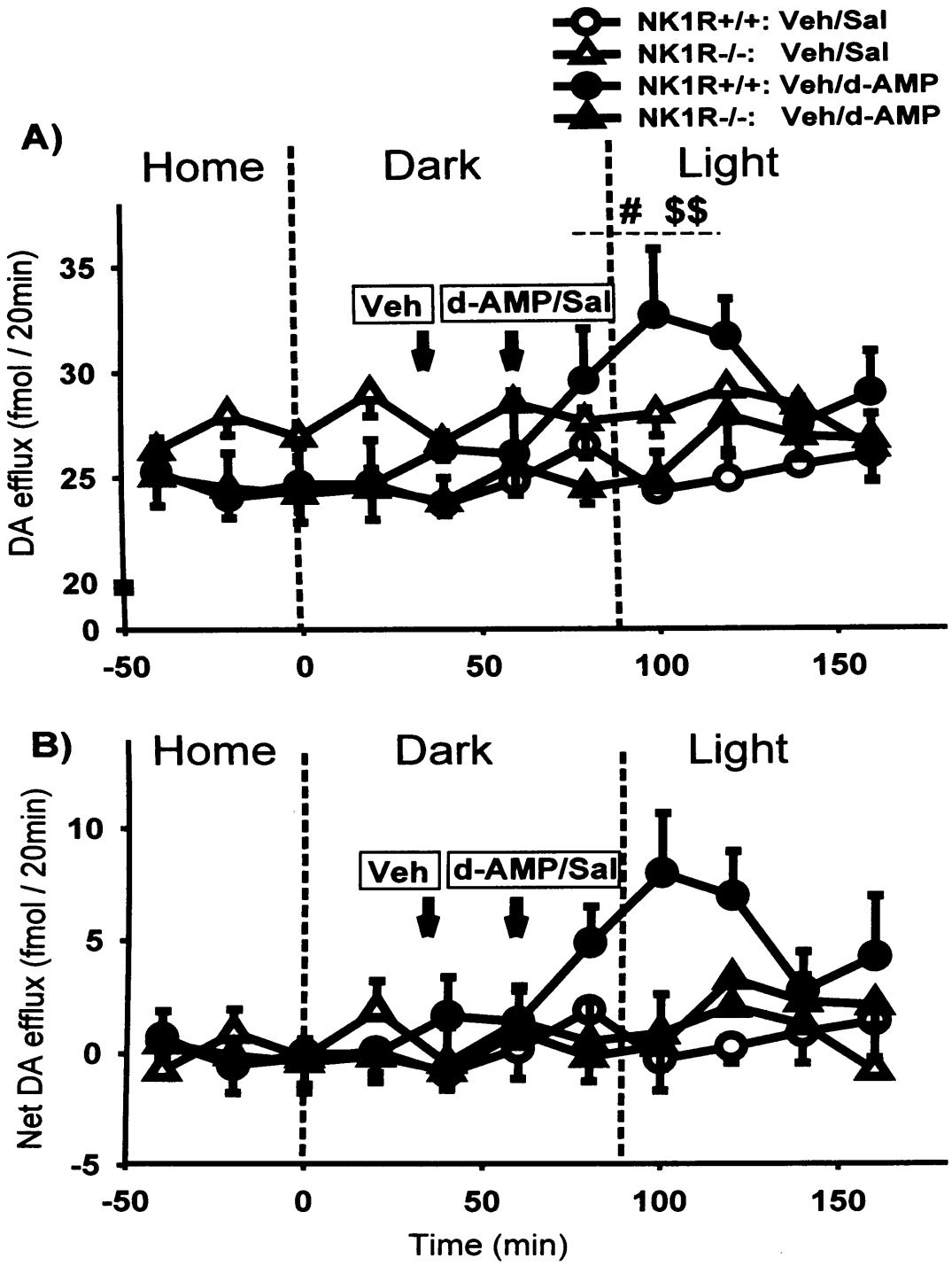


Fig 6.4 A) Raw data and B) the net changes showing the effects of *d*-AMP on DA efflux in the dorsal striatum of NK1R+/+ and NK1R-/- mice in the LDEB (N = 3~4/group). RP: PR 67580; Sal: saline; Veh: vehicle. Statistics were performed using the raw data: NK1R+/+ (Veh/*d*-AMP): T-40~0 *vs.* T80~120: # $P < 0.05$. At T80~120, NK1R+/+ (Veh/*d*-AMP) *vs.* NK1R+/+ (Veh/Sal): $$ $P < 0.01$.

To evaluate the effect of the NK1R antagonist, RP 67580, on the dorsal striatal DA response to *d*-AMP in the two genotypes, animals that received treatments of 'vehicle / *d*-AMP' and 'RP 67580 / *d*-AMP' were compared.

It was observed that the effects of RP 67580 differed in the two genotypes:

'Genotype * RP 67580 * Bin' interaction:	$F_{1,13} = 11.7$	$P < 0.01$
'Genotype * RP 67580' interaction:	$F_{1,13} = 11.7$	$P < 0.01$

Specifically, whereas pretreatment with RP 67580 had no effect in NK1R-/- mice administered with *d*-AMP, it abolished the increase in the striatal DA response of NK1R+/+ mice to the psychostimulant. However, this was only apparent when comparing the net changes of efflux in the wild-type ($F_{1,6} = 13.0$, $P < 0.05$; see Fig 6.5 B).

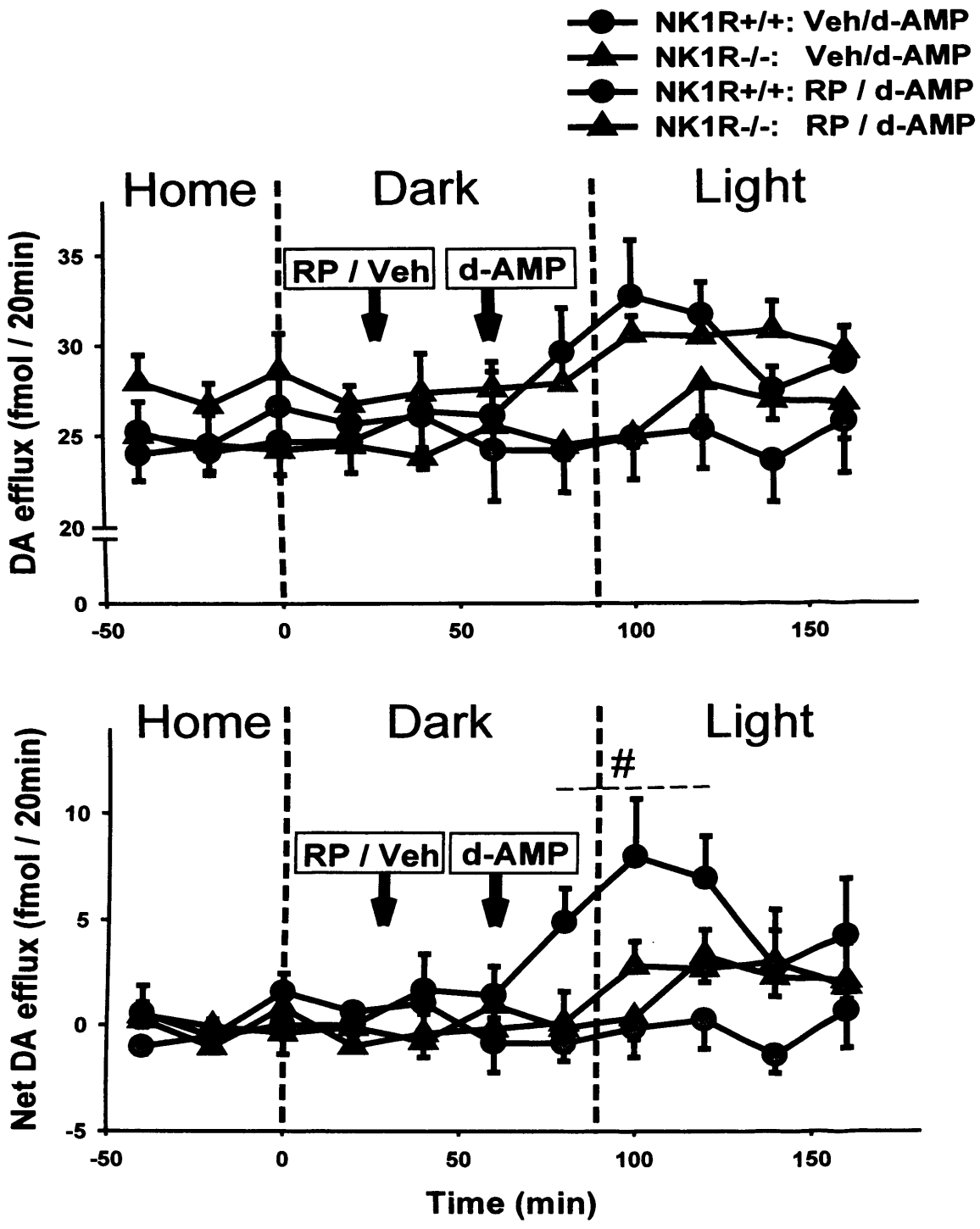


Fig 6.5 A) Raw data and B) net changes showing the effects of RP 67580 on the *d*-AMP-induced changes in DA efflux in the dorsal striatum of NK1R+/+ and NK1R-/- mice in the LDEB (N = 3~4/group). RP: PR 67580; Veh: vehicle. Statistics were performed on the net changes: At T80~120, NK1R+/+ (RP/*d*-AMP) *vs.* NK1R+/+ (V//*d*-AMP): # $P < 0.05$.

6.4.2. DA efflux in the PFC of NK1R+/+ and NK1R-/- mice in the LDEB

To investigate changes in PFC DA efflux, a similar 5-way ANOVA, as described in Section 6.4.1, was carried out among all 8 groups. Here, basal efflux was compared with Bin T120~160, as this is when efflux had stabilized.

The results showed a 'Genotype * RP 67580 * bin' interaction ($F_{1, 18} = 6.7$, $P < 0.05$). This indicated that effects of the NK1R antagonist, RP 67580, differed in the two genotypes.

Again, the data reported in this section come from the same fully randomized study, but were discussed in two sub-sections (see Fig 6.6 ~ 6.8) for clarity.

- ***The effects of NK1R antagonism on basal DA efflux in the PFC***

As reported in Section 4.4.4, basal PFC DA efflux was found to be lower in NK1R-/- (14.1 ± 1.3 fmol / 20min) than in NK1R+/+ mice (27.2 ± 2.0 fmol / 20min) ($F_{1, 18} = 181.2$, $P < 0.001$).

This genotype difference in cortical DA efflux was abolished by pretreatment with the NK1R antagonist, RP 67580, which reduced efflux in NK1R+/+, but not NK1R-/- mice ('Genotype * RP 67580' interaction: $F_{1, 18} = 5.9$, $P < 0.05$; see Fig 6.6).

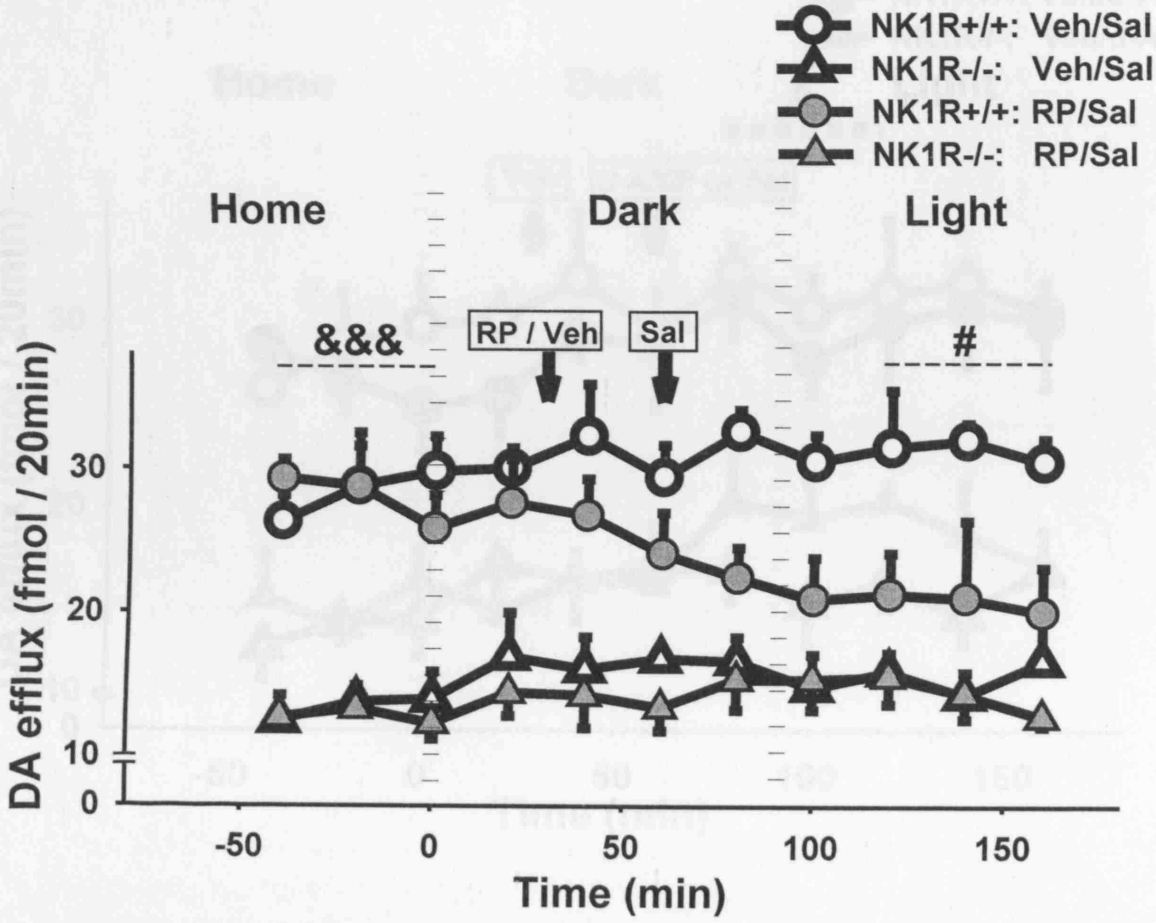


Fig 6.6 The effect of the NK1R antagonist, RP 67580, on basal DA efflux in the PFC of NK1R+/+ and NK1R-/- mice (N = 3~4/group). RP: PR 67580; Veh: vehicle. Genotype difference in basal DA efflux: &&& $P < 0.001$. At T120~160, NK1R+/+ (RP/Sal) *vs.* NK1R+/+ (Veh/Sal): # $P < 0.05$.

- ***The effects of NK1R antagonism on the PFC DA response to d-AMP***

To investigate the effects of *d*-AMP on PFC DA efflux, basal efflux was compared with Bin T80~120.

Acute administration of *d*-AMP did not affect cortical DA efflux in either genotype, although a main effect of 'Bin' was found ($F_{1,9} = 16.1$, $P < 0.01$) (see Fig 6.7).

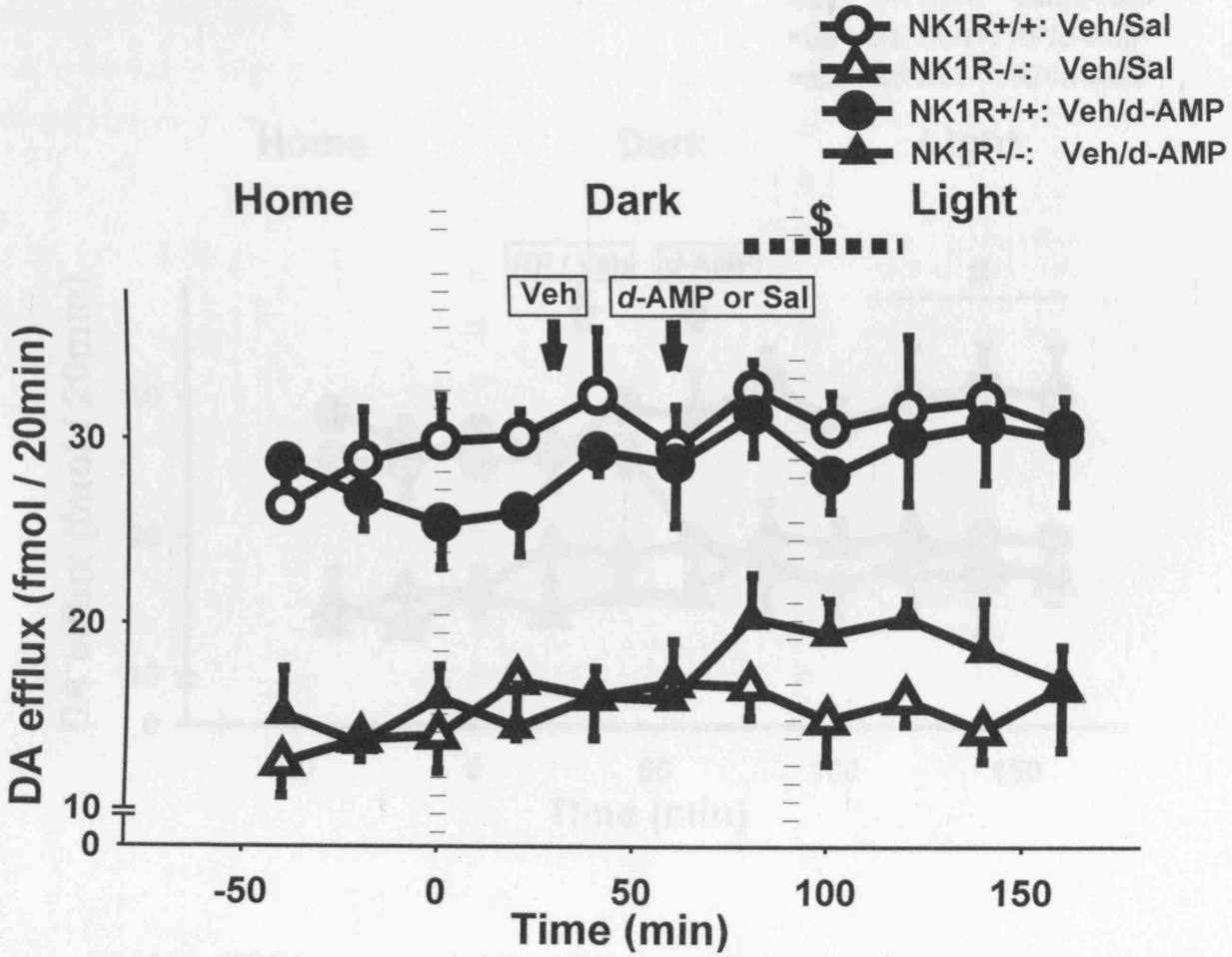


Fig 6.7 The effects of *d*-AMP on DA efflux in the PFC of NK1R+/+ and NK1R-/- mice in the LDEB (N = 3~4/group). RP: RP 67580; Sal: saline; Veh: Vehicle.
Main effect of 'Bin': T80~120 *vs.* Basals: $ $P < 0.05$.

The effect of the NK1R antagonist, RP 67580, was similar in the *d*-AMP-treated animals (see Fig 6.8) as in the saline-treated ones (see Fig 6.6): After pretreatment with RP 67580, PFC DA efflux rapidly decreased in NK1R+/+ mice to that in the knockout ($F_{1,6} = 7.6$, $P < 0.05$). In contrast, NK1R antagonism had no effect in NK1R-/- mice.

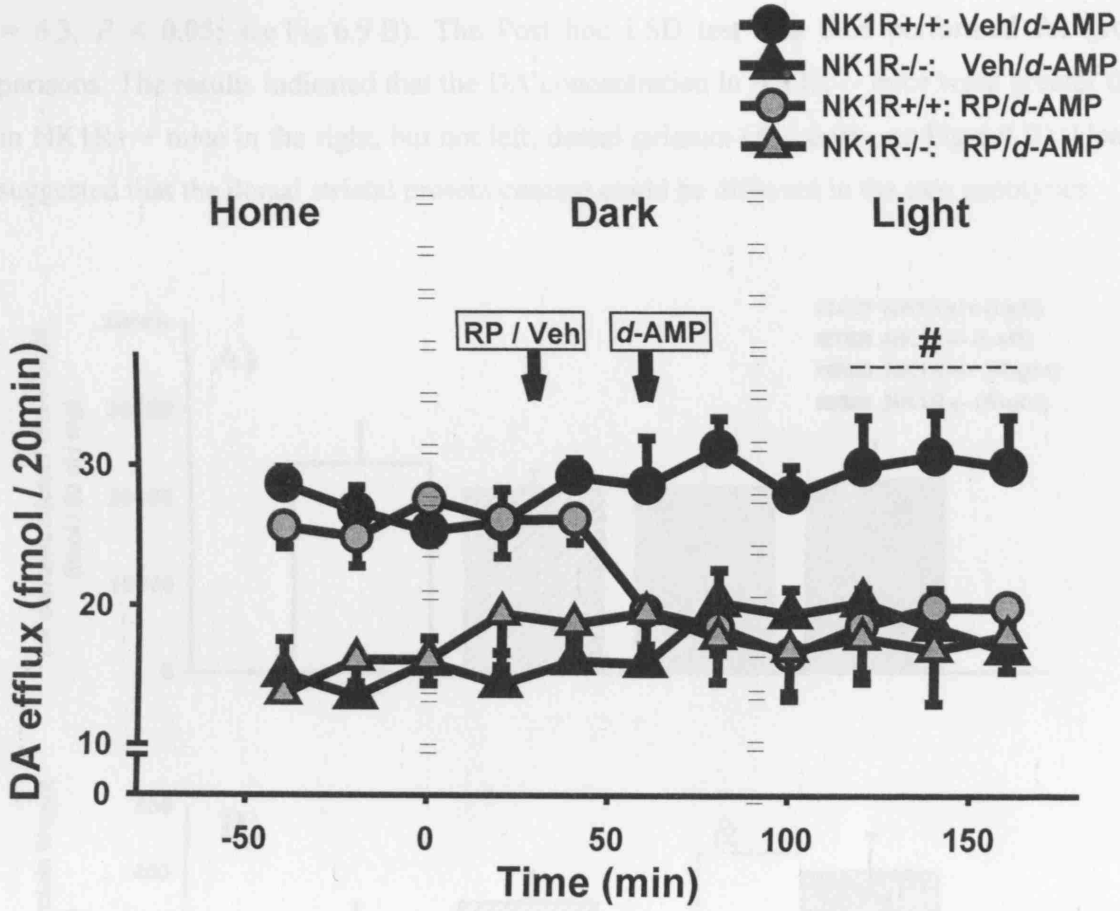


Fig 6.8 The PFC DA response to *d*-AMP in NK1R+/+ and NK1R-/- mice pretreated with the NK1R antagonist, RP 67580 (N = 3~4/group). RP: RP 67580; Sal: saline; Veh: Vehicle. At T120~160, NK1R+/+ (RP/ *d*-AMP) *vs.* NK1R+/+ (Veh/ *d*-AMP): # $P < 0.05$.

6.4.3. DA and protein concentrations in the mouse striatal homogenates

- ***DA concentration in the dorsal striatum***

When comparing the striatal DA concentration per *unit wet weight*, there was no difference between genotype or side of the brain (see Fig 6.9 A). However, when comparing striatal DA concentrations *per unit weight of protein*, 2-way ANOVA revealed a main effect of genotype ($F_{1, 35} = 6.3$, $P < 0.05$; see Fig 6.9 B). The Post hoc LSD test was then performed for group comparisons. The results indicated that the DA concentration in NK1R-/- mice were greater than that in NK1R+/+ mice in the right, but not left, dorsal striatum ($P < 0.05$; see Fig 6.9 B). Hence, this suggested that the dorsal striatal protein content could be different in the two genotypes.

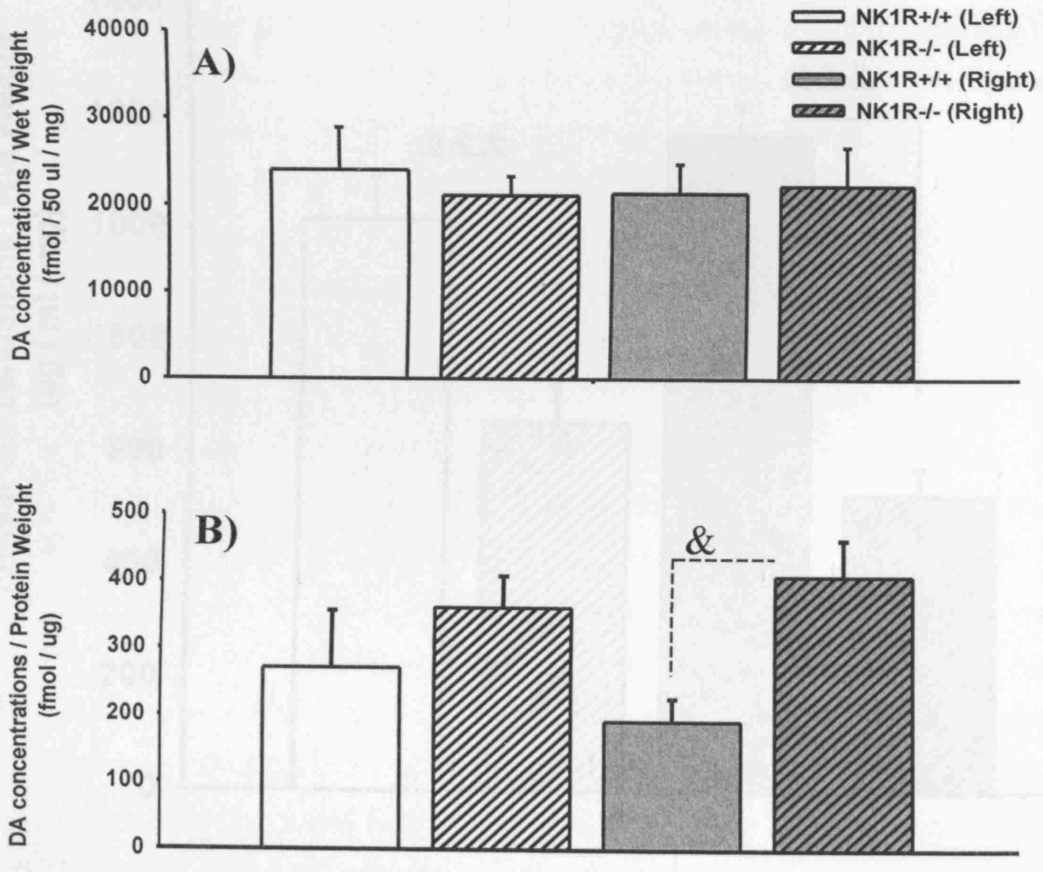


Fig 6.9 The DA concentration A) per mg of wet tissue and B) per μg of protein in the dorsal striatum of NK1R+/+ and NK1R-/- mice (N = 9~10/group). NK1R+/+ *vs.* NK1R-/-: & $P < 0.05$.

- ***Protein concentration in the dorsal striatum***

2-way ANOVA showed a main effect of genotype ($F_{1, 35} = 53.7$, $P < 0.001$) as well as 'Genotype * Side' interactions ($F_{1, 35} = 4.0$, $P < 0.05$). Comparisons between groups, using the Post-Hoc LSD test, revealed that protein concentration / wet weight was lower in NK1R-/- than in NK1R+/+ mice in both the left ($P < 0.001$) and the right striatum ($P < 0.001$) (see Fig 6.10).

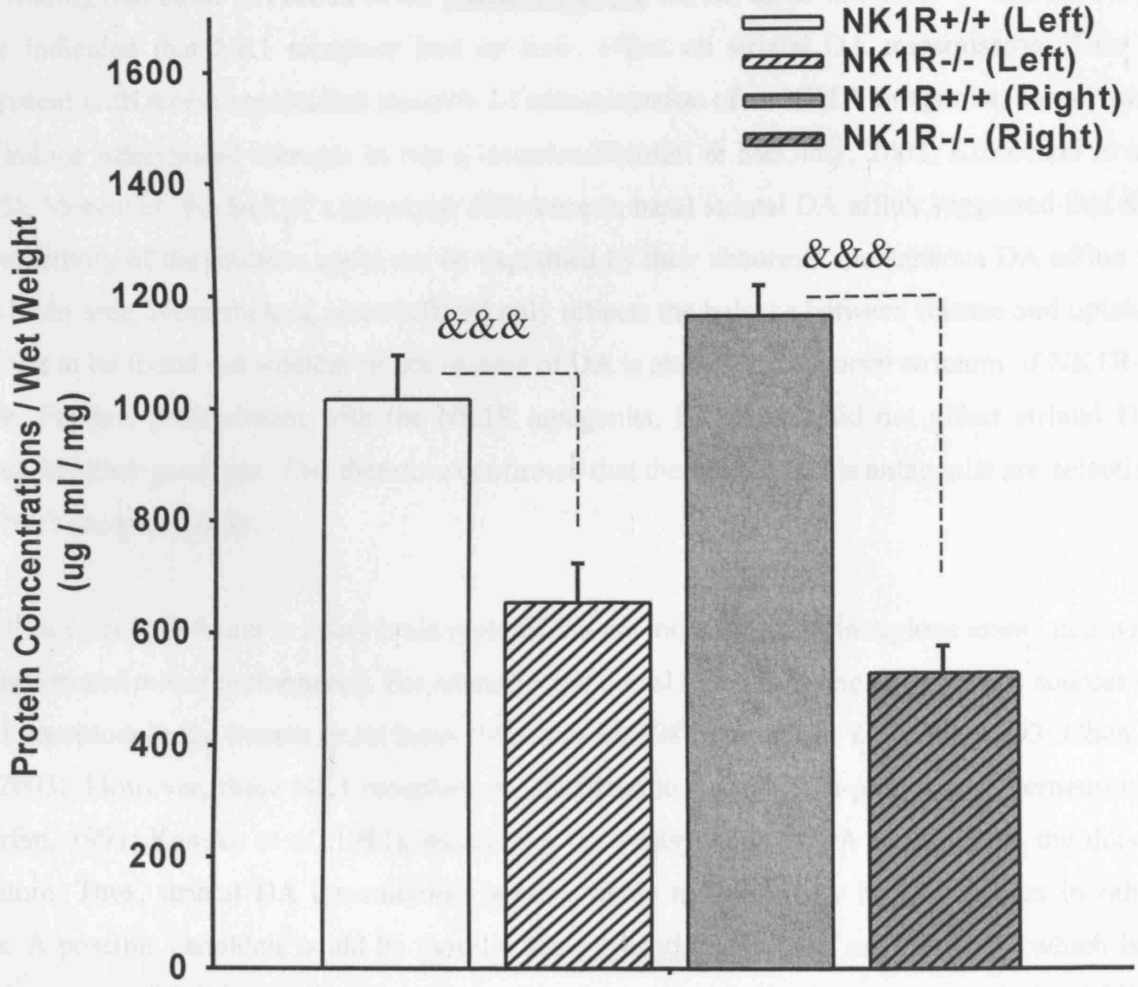


Fig 6.10 Protein concentrations per mg of wet tissue in the dorsal striatum of NK1R+/+ and NK1R-/- mice (N = 9~10/group). NK1R+/+ *vs.* NK1R-/-: &&& $P < 0.001$

6.5. Discussion

The current findings revealed that basal DA efflux in the PFC and the dorsal striatal DA response to *d*-AMP are abnormal in NK1R-/- mice. Moreover, the mutants also have lower protein concentration per mg of wet tissue in their striatal homogenates.

6.5.1. Basal DA efflux in the dorsal striatum & the PFC

The finding that basal DA efflux in the **dorsal striatum** did not differ in NK1R+/+ and NK1R-/- mice indicated that NK1 receptors had no tonic effect on striatal DA transmission. This is consistent with recent reports that intrastriatal administration of an NK1R antagonist, alone, does not induce behavioural changes in rats (Gonzalez-Nicolini & McGinty; 2002; Krolewski *et al*, 2005). Moreover, the lack of a genotype difference in basal striatal DA efflux suggested that the hyperactivity of the mutants could not be explained by their abnormal spontaneous DA efflux in this brain area. Nevertheless, since 'efflux' only reflects the balance between release and uptake, it is yet to be found out whether or not *release* of DA is altered in the dorsal striatum of NK1R-/- mice. Further, pretreatment with the NK1R antagonist, RP 67580, did not affect striatal DA efflux in either genotype. This therefore confirmed that the effects of this antagonist are selective for NK1 receptors, only.

NK1 receptors are found in many brain regions, but are most abundant in regions associated with cognition and motor performance. For example, the dorsal striatum is one of the major sources of NK1 receptors in the human or rat brain (Shults *et al*, 1984; Otsuka & Yoshioka, 1993; Chen *et al*, 2003). However, these NK1 receptors are localized to striatal ACh-producing interneurones (Gerfen, 1991; Kaneko *et al*, 1993), hence they are postsynaptic to DA terminals in the dorsal striatum. Thus, striatal DA transmission is more likely modulated by NK1 receptors in other sites. A possible candidate could be those in the substantia nigra (SN; see Fig 6.11), which is a major source of DA in the dorsal striatum. Despite much debate, the existence of nigral NK1 receptors has been demonstrated using *in situ* hybridization and immunohistochemical methods (Whitty *et al*, 1995; Futami *et al*, 1998). Other microdialysis studies (e.g. Reid *et al*, 1991) have

lent further support to the effect of nigral NK1 receptors by showing that local infusion of substance P in the SN augments striatal DA transmission.

DA transmission in the dorsal striatum might also be regulated by NK1 receptors in the DRN and the LC, particularly the latter nucleus where NK1 receptors are abundant (Shults *et al*, 1984; Otsuka & Yoshioka, 1993; Chen *et al*, 2000; see Fig 6.11). Using a retrograde axonal transport method, the dorsal striatum has been shown to be innervated by 5-HT neurones in the DRN, where NK1 receptors are in the cytoplasm of 5-HT neurones (Lacoste *et al*, 2006) as well as colocalized with GABA interneurones (Ma & Bleasdale, 2002). This 5-HT innervation of the dorsal striatum can be either direct (Pasquier *et al*, 1977; Snyder *et al*, 1986), or indirect *via* the substantia nigra (Corvaja *et al*, 1993). Moreover, there is immunohistochemical (Simon *et al*, 1979) and behavioural evidence (Donaldson *et al*, 1976; Płaźnik *et al*, 1983) that the LC projects directly to the SN and the DRN, which could also indirectly regulate DA transmission in the dorsal striatum.

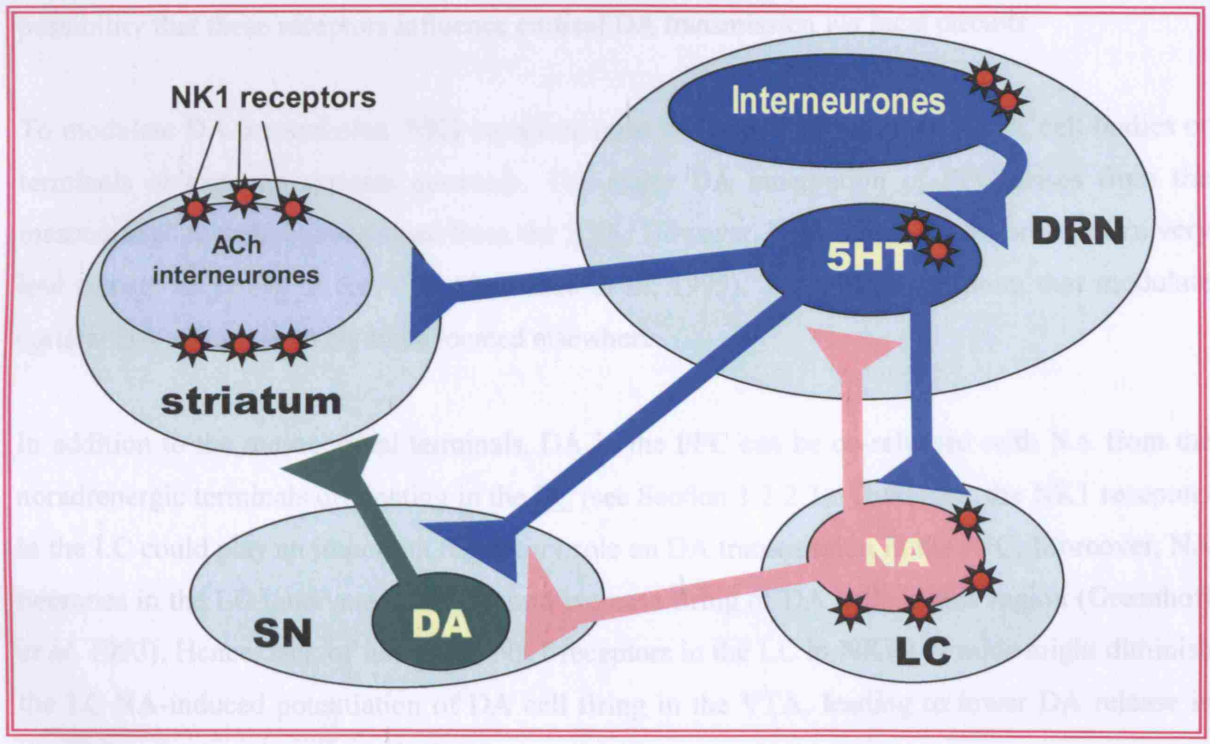


Fig 6.11 Diagram illustrating possibilities for modulation of DA transmission in the dorsal striatum by NK1 receptors (DRN: dorsal raphe nucleus; LC: locus coeruleus; SN: substantia nigra).

Unlike basal DA efflux in the dorsal striatum, that in the **PFC** of NK1R-/- mice was again found to be less than that of NK1R+/+ mice, as reported in Section 4.4.4. This might explain why striatal DA efflux did not differ in the two genotypes, given that PFC DA has an inhibitory effect on DA transmission in the subcortical areas (see Section 1.2.3.2). Nevertheless, the striatal DA transmission in NK1R-/- mice was not greater than that in NK1R+/+ mice, either, possibly due to increased expression and / or activity of striatal DATs in the knockout (see Section 6.5.2). The genotype difference in basal PFC DA efflux was abolished by pretreatment with the NK1R antagonist, RP 67580, which reduced cortical DA efflux in NK1R+/+ mice, only. This suggested that the maintenance of basal PFC DA transmission requires functional NK1 receptors.

The explanation of modulation of DA transmission in the PFC by NK1 receptors is unknown, as yet. A moderate density of NK1 receptors has been revealed in human PFC and visual cortex (Tooney *et al*, 2000). However, these receptors are *postsynaptic* to catecholamine terminals, on small non-pyramidal cells and in the neuropil (layers I-III). This therefore makes it unlikely that DA efflux in the PFC is modulated by local NK1 receptors, although we could not rule out the possibility that these receptors influence cortical DA transmission *via* local circuits.

To modulate DA transmission, NK1 receptors must be located on presynaptic DA cell bodies or terminals or even on systems upstream. The major DA innervation of PFC arises from the mesocortical projection, originated from the VTA. However, NK1 receptors are present at a very low density (if at all) in the VTA (Seabrook *et al*, 1995). Thus, NK1 receptors that modulate cortical DA efflux are likely to be located elsewhere.

In addition to the mesocortical terminals, DA in the PFC can be co-released with NA from the noradrenergic terminals originating in the LC (see Section 1.2.2.3). Therefore, the NK1 receptors in the LC could play an important regulatory role on DA transmission in the PFC. Moreover, NA neurones in the LC innervate the VTA, and increase firing of DA cells in this region (Greenhoff *et al*, 1993). Hence, lack of functional NK1 receptors in the LC in NK1R-/- mice might diminish the LC-NA-induced potentiation of DA cell firing in the VTA, leading to lower DA release in the PFC in the knockout (see Fig 6.12).

It is also possible that the DRN 5-HT system is involved. In NK1R-/- mice, 5-HT release is augmented (Froger *et al*, 2001). This could lead to increased inhibition of DA neurones in the VTA, by activating the excitatory 5-HT_{2C} receptors on the GABA interneurones in this midbrain area (Bubar & Cunningham, 2007). This might lead to lower DA release from the mesocortical terminals in the PFC in the mutants. Moreover, the LC and the DRN send reciprocal projections to each other: whereas the LC exerts an excitatory influence on 5-HT efflux in the DRN (Pudovkina *et al*, 2002), the DRN inhibits the LC NA firing (Chiang & Aston-Jones, 1993; Szabo & Blier, 2001). The DRN 5-HT system also reduces the PFC NA response to local infusion of *d*-AMP in rats (Géranton *et al*, 2003). Thus, these two nuclei appear to form a self-regulating loop, in which NK1 receptors exquisitely modulate cortical DA efflux (see Fig 6.12).

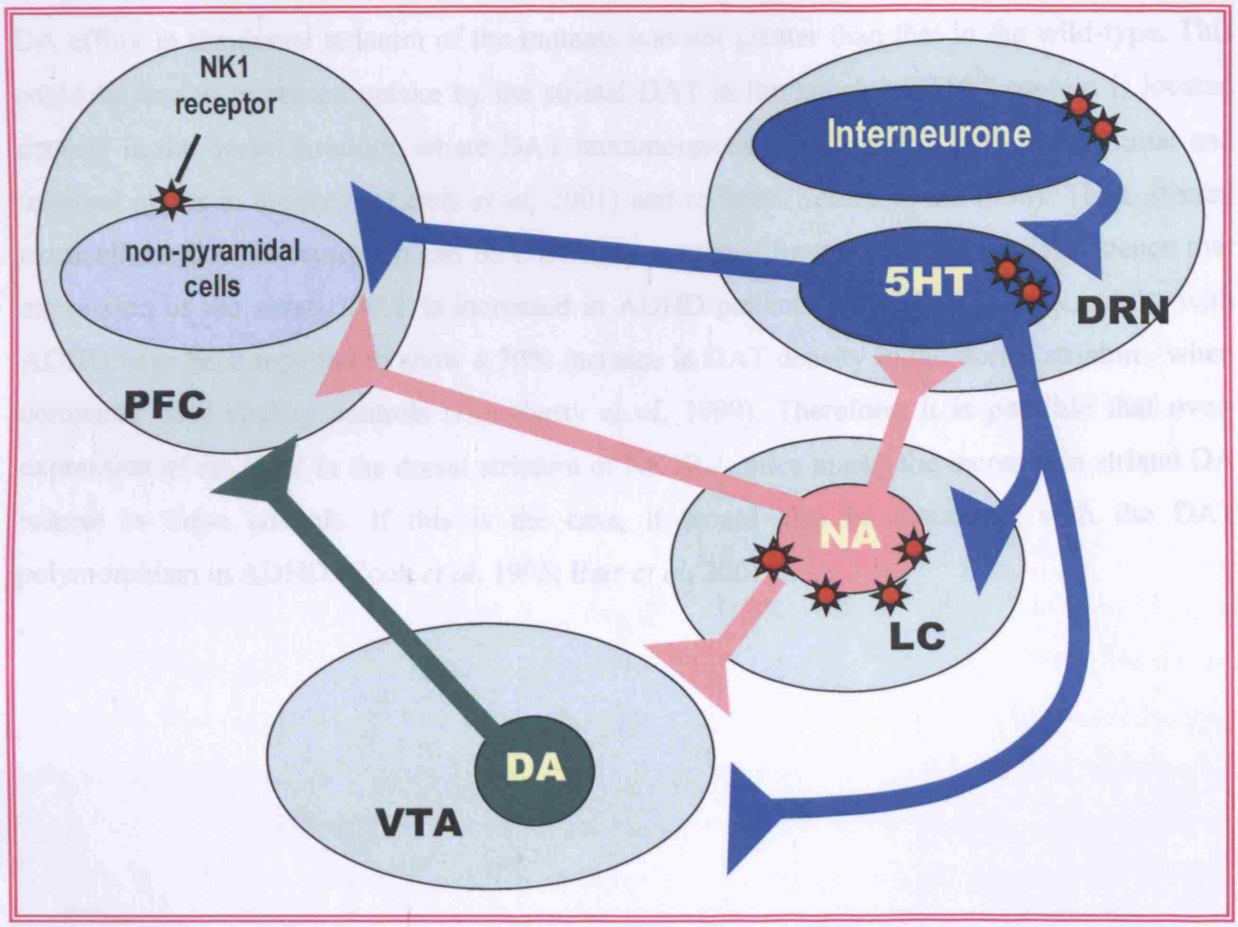


Fig 6.12 Diagram illustrating possibilities for modulation of DA transmission in the PFC by NK1 receptors (DRN: dorsal raphe nucleus; LC: locus coeruleus; VTA: ventral tegmental area).

6.5.2. Basal striatal & PFC DA efflux in NK1R-/- mice: relevance to ADHD

As discussed in Section 4.5.4, the lower DA efflux in the PFC of NK1R-/- mice could echo the hypofrontality of ADHD patients. Moreover, the finding of a genotype difference in DA efflux in the PFC, but not the dorsal striatum, is in keeping with the report from Ernst *et al* (1998). These authors found that a reduction of DOPA decarboxylase activity and DA storage processes in the PFC, but not the dorsal striatum, in patients with ADHD (as reflected by 50% lower ratio of specific to nonspecific radioactivity of the DA tracer, [fluorine-18]DOPA, in the former brain region). This suggested that ADHD is associated with reduced DA transmission in the PFC, only.

Despite the reduced PFC inhibition of striatal DA transmission in NK1R-/- mice (*cf.* NK1R+/+), DA efflux in the dorsal striatum of the mutants was not greater than that in the wild-type. This could be due to increased uptake by the striatal DAT in the knockout. DAT protein is located densely in the dorsal striatum, where DAT immunoreactivity is found in both pre-terminal and terminal axons in monkeys (Lewis *et al*, 2001) and rodents (Sesack *et al*, 1998). Thus, striatal extracellular DA concentration can be efficiently regulated by the DAT. There is evidence that expression of the striatal DAT is increased in ADHD patients. In a SPECT study, adults with ADHD have been reported to show a 70% increase in DAT density in the dorsal striatum, when compared with healthy controls (Dougherty *et al*, 1999). Therefore, it is possible that over-expression of the DAT in the dorsal striatum of NK1R-/- mice masks the increase in striatal DA release in these animals. If this is the case, it would also be consistent with the DAT polymorphism in ADHD (Cook *et al*, 1995; Barr *et al*, 2001)

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6.5.3. Modulation of the striatal & the PFC DA responses to *d*-AMP by NK1 receptors

In the dorsal striatum, *d*-AMP increased DA efflux in NK1R+/+, but not NK1R-/-, mice. This genotype difference was, again, abolished by pretreatment with the NK1R antagonist, RP 67580, which had no effects in the mutants. Hence, these results indicated that the striatal DA response to *d*-AMP needs functional NK1 receptors.

It is thought that the *d*-AMP-induced motor arousal is mostly attributed to the drug-induced increase in NAc DA transmission (Kelly *et al*, 1975; Wise, 1996). However, there is evidence that the locomotor response to *d*-AMP did not differ in rats whose NAc is lesioned with kainic acid, and in sham-operated rats (Kafetzopoulos, 1986). This indicated that the locomotor stimulating effect of *d*-AMP is not exclusively dependent on DA in the NAc, but also on other brain regions. The finding that *d*-AMP increased locomotor activity of NK1R+/+ mice (see Chapter 5) and their DA efflux in the dorsal striatum (see this Chapter) suggested that striatal DA transmission could, at least in part, contribute to the animals' motor response to *d*-AMP. In contrast, the psychostimulant did not increase the motor response of NK1R-/- mice or that of the wild-type treated with the NK1R antagonist, RP 67580 (see Chapter 5), and did not affect their DA efflux in the dorsal striatum (see this Chapter). These results therefore suggested that the striatal DA response to *d*-AMP requires functional NK1 receptors. This is in agreement with the study by Gonzalez-Nicolini & McGinty (2002), who showed that intrastriatal infusion of a selective NK1R antagonist in rats decreases their motor response to systemic administration of *d*-AMP. Taken together, all these studies support the current finding that NK1 receptors are required to rally a DA response to *d*-AMP in the dorsal striatum.

There is also evidence that the stimulating effect of *d*-AMP needs activation of α_1-adrenoceptors (possibly) in the PFC (Dickinson *et al*, 1988; Blanc *et al*, 1994; Darracq *et al*, 1998). Tassin and colleagues have investigated this in detail in freely-moving rats. Using *in vivo* microdialysis, they found that both systemic (i.p.) injection and local infusion of *d*-AMP raised DA efflux in the NAc, but only i.p. injection of the drug increased locomotion. Further, i.p. injection of the selective α_1-antagonist, prazosin, inhibits the hyperactivity caused by systemic *d*-AMP, but does not modify the NAc DA response to *d*-AMP (Darracq *et al*, 1998). They therefore suggested that the *d*-AMP-induced increase in NAc DA efflux could be divided into a major nonfunctional DA

component (caused by the local effect of *d*-AMP in the NAc) which does not lead to locomotor hyperactivity, and a smaller functional DA component (caused by an effect of *d*-AMP distal from the NAc) which correlated with the development of hyperactivity. Activation of PFC α_1-adrenoceptors is needed to elicit the *d*-AMP-induced hyperactivity, as these receptors control the functional part of released DA, either directly by modifying release of glutamic acid (which co-occurred with release of DA) in the NAc, or indirectly by increasing VTA DA neuronal firing and hence triggering release of DA in the NAc (Darracq *et al*, 1998). Collectively, these studies indicated that PFC plays a vital role in the motor stimulating effects of *d*-AMP.

Over the years, various microdialysis studies in rats and mice have revealed that *d*-AMP enhances DA transmission in the PFC. However, the current study reported a lack of cortical DA response to *d*-AMP in NK1R+/+ and NK1R-/- mice. This could be due to: (i) efficient clearance processes, given that uptake *via* membrane-bound transporters is the primary mechanism for terminating monoamine action and maintaining monoamine homeostasis (Elsworth & Roth, 1997; Géranton *et al*, 2003a); (ii) some factor(s) might be holding down a cortical PFC DA response to the drug. We are not the first group who found no increase in PFC DA efflux by *d*-AMP. Hedou *et al* (2001) even showed a *d*-AMP-induced *decrease* in cortical DA transmission in rats. In fact, comparisons across different studies should be done with caution, due to disparities of various experimental factors that could influence the results. For example, most of these investigations (see Table 6.1) dialyzed different parts of the PFC from our group. Some studies used guide cannulae, but we do not. Moreover, a higher Ca^{2+} concentration was used in the perfusion medium in some cases (e.g. Ventura *et al*, 2004; Engelman *et al*, 2006; Schank *et al*, 2006). All these and other disparities between experimental procedures might explain the different findings between the current study and the previous ones.

	References	Guide Cannula	Dialysis Window (mm)	Flow rate (µl / min)	[Ca^{2+}] in perfusion medium (mM)	PFC co-ordinates (mm)	Region of PFC	Basal DA efflux	Basal DA efflux equivalent (fmol/20µl)	*d*-AMP - induced changes in DA efflux	Strain	Dose of *d*-AMP (mg/kg)	lower sensitivity limit	Surgery recovery time before dialysis
mouse	The current study	No	1	1.5	1.3	AV: 2.1 ML: 1.0 DV: -2.0	M2	10~30 fmol/30µl	7 ~ 20	None	C57BL/6	2.5 (i.p.)	5~8 fmol	1 day
	Schank *et al* (2006)	Yes	2	2	2.2	AV: 2.5 ML: 0.6 DV: -1.0	M2 + VO	0.5 pg/20µl	2.6	+75~ 200%	129/SvEv + C57BL/6	2.5 (i.p.)	0.1 pg/20µl (0.5 fmol /20 µl)	2~3 days
	Ventura *et al* (2004)	Yes	2	2	2.2	AV: 2.5 ML: 0.6 DV: -1.0		0.29 pg/20µl	1.5	+150%	C57BL/6	2.5 (i.p.)	N/A	2 days
rat	Engelman *et al* (2006)	No	2.5 (Loop)	1	2.5	AV: 3.2 ML: 0.7 DV: -2.0	Cg1	2~5 nM/20µl	40 ~ 100	+250%	Wistar	1.0 (i.p.)	0.1 nM (2 fmol)	2 days
	Shoblock *et al* (2004)	Yes	2	1	1.2	AV: 3.2 ML: 0.1 DV: -6.1	DP	0.69 pg/10µl	7.3	+600%	Sprague-Dawley	2.0 (i.p.)	N/A	4 days
	Balla *et al* (2003)	Yes	4	1	1.2	AV: 4.1 ML: 1.0 DV: -1.2	Fr2, Cg1, Cg3, VLO	0.5~2 pg/10µl	5.2 ~ 21	+200~ 300%	Sprague-Dawley	1.0 (s.c.)	N/A	1 day
	Dalley *et al* (2002)	No	2	1.5	1.3	AV: 3.0 ML: 1.2 DV: -4.0	Cg3	1.3 fmol/ 22.5µl	1.27	+900%	Lister Hooded	0.125 (i.v.)	N/A	2 days *
	Berridge & Stalnaker (2002)	No	4	1.5	1.3	AV: 3.2 ML: 1.0 DV: -5.0	IL	0.89 pg/20µl	4.7	+200%	Sprague-Dawley	0.15 (s.c.)	0.3 pg/20µl (1.6 fmol /20 µl)	1 day **
	Hedou *et al* (2001)	Yes	N/A	1.0	1.2	(d)^ (v)^ AV: 2.7; 3.2 ML: 0.5; 0.5 DV: -4.0; -6.0	Cg3 (d)^ DP (v)^	in pg/µl: 0.4 (d)^ 1.7 (v)^	42 (d)^ 180 (v)^	-60% (d)^ -70% (v)^	Wistar	1.5 (i.p.)	N/A	7 days

Table 6.1. Microdialysis studies that investigated the effects of systemic aministration of *d*-AMP on DA efflux in the PFC of freely-moving rats and mice.
Subregions in the PFC: M2 (Secondary motor cortex); VO (Ventral orbital cortex); VLO (Ventral lateral orbital cortex); PrL (Prelimbic cortex); Cg1 (Cingulate cortex area 1); Cg3 (Cingulate cortex area 3); DP (Dorsal peduncular cortex); Fr2 (Frontal cortex area 2; equivalent to the mouse M2 region); IL (Infralimbic cortex)

*: Surgery was performed 6 weeks after behavioural training on the 5-choice serial reaction time task.

**: Prior to probe insertion, an electroencephalographic (EEG) electrode was implanted into contralateral frontal cortex (in mm: AV 3.0; ML 1.5; DV -5), and an electromyographic (EMG) electrode was implanted into the dorsal neck muscle.

(d)^: dorsal PFC (i.e. anterior cingulated)

(v)^: ventral PFC (i.e. infralimbic cortex)

6.5.4. The DA response of NK1R-/- mice to *d*-AMP: relevance to ADHD

In NK1R+/+ mice, the increase in dorsal striatal DA efflux by *d*-AMP could help explain why this drug causes motor activation in these animals in the LDEB test (see Section 5.4.1) and arousal in healthy humans. The finding that *d*-AMP increased DA efflux in the dorsal striatum, but not that in the PFC, of the wild-type is consistent with the microdialysis study by Mazei *et al* (2002), who revealed that stimulants increase DA release to a greater extent in the striatum than the cortex. This regional difference could be due to greater density of the DAT in the subcortical area (Sesack *et al*, 1998; Cragg *et al*, 2002), which is inhibited by psychostimulants.

In contrast, *d*-AMP did not alter striatal DA transmission in NK1R-/- mice. This might be why this drug did not increase motor arousal in the mutants (see Section 5.4.1) or in individuals with ADHD. The lack of a striatal DA response to *d*-AMP in NK1R-/- mice concurs with the findings in the SHR, as electrical stimulation-evoked release of DA is also deficient in the dorsal striatum of these rats (Linthorst *et al*, 1990; Russell *et al*, 1995, 1996).

Nevertheless, the current data do not explain why both *d*-AMP and MPH, the two most commonly used drugs in ADHD, ***reduced*** hyperactivity of NK1R-/- mice to the levels of vehicle-treated NK1R+/+ mice. Further investigations are therefore needed to address this issue.

6.5.5. Striatal DA & protein concentrations in the mouse striatal homogenates

There was no genotype difference in DA concentration *per mg of wet tissue* in either side of the dorsal striatum. However, DA concentration *per μg of protein* was greater in NK1R-/- than in NK1R+/+ mice in the right dorsal striatum. This was due to a decrease in the concentration of protein per mg of wet tissue in NK1R-/- mice, rather than an increase in their DA content.

Reduced protein concentration in the dorsal striatum of NK1R-/- mice consolidates their validity as a model of ADHD. This is because neuroimaging studies reported that people with ADHD have a smaller caudate size, and a loss of the usual caudate asymmetry, despite the inconsistent reports on the lateralization of this abnormality (Hynd *et al*, 1993; Filipek *et al*, 1997; Castellanos *et al*, 1994, 1996, 2001, 2002; Mataró *et al*, 1997). The current data revealed that,

compared with NK1R+/+ mice, striatal protein concentrations were lower on both sides in NK1R-/- mice. The reduction could be more apparent in the right dorsal striatum, as there was a 'Genotype * Side' interaction: lower in the right than left in NK1R-/- mice, and the opposite in NK1R+/+ mice, although significance was not reached when comparing sides in NK1R-/- mice. So far, the striatal lateralization investigations are all based on human subjects. Hence the current study could be the first one addressing this issue in rodents. Despite the fact that a lower protein concentration does not directly conclude a smaller caudate volume in NK1R-/- mice, it implies a reduction of the number of cells in this brain region of the mutants. Further neuroimaging studies could be carried out to compare the size of the dorsal striatum in NK1R+/+ and NK1R-/- mice.

6.6. Summary

- **In the dorsal striatum:**
  - **Basal DA efflux did not differ in NK1R+/+ and NK1R-/- mice, and the NK1R antagonist, RP 67580, had no effects in either genotype.**
    --NK1 receptors had no tonic effect on basal DA efflux in the dorsal striatum.
    --Confirmed the selectivity of this NK1R antagonist.
  - ***d*-AMP increased striatal DA efflux in NK1R+/+, but not NK1R-/- mice or NK1R+/+ mice given the NK1R antagonist, RP 67580.**
    --The increase in striatal DA efflux by *d*-AMP requires functional NK1 receptors.
    --Possibly explaining why psychostimulants increase arousal in NK1R+/+ mice and normal humans, but not in NK1R-/- mice or ADHD patients.

- **In the PFC:**
  - **Basal DA efflux was lower in NK1R-/- than in NK1R+/+ mice. The NK1R antagonist, RP 67580, abolished this genotype difference by reducing DA efflux in NK1R+/+ mice, only.**
    --NK1 receptors are required to maintain basal PFC DA transmission.
    --The PFC DA deficit in NK1R-/- mice might echo hypofrontality of ADHD.
  - ***d*-AMP did not alter cortical DA efflux in NK1R+/+ or NK1R-/- mice.**
    This could be due to efficient clearance processes and / or some factor(s) holding down a PFC DA response to *d*-AMP in this brain region.
- **DA & protein concentrations in the dorsal striatal homogenates:**
  - **DA concentration per μg of protein is greater in NK1R-/- than in NK1R+/+ mice in the right dorsal striatum.**
    --This seems to be due to a reduction of protein concentration per mg of wet striatal tissue, rather than an increase in DA content, in the mutants.
    --Consisted with a smaller caudate size in ADHD patients.

In the next chapter, the validity of NK1R-/- mice as an ADHD model was further tested by investigating the PFC NA response to systemic injection of *d*-AMP in NK1R+/+ and NK1R-/- mice in the LDEB.

Chapter 7. Abnormal noradrenergic response in the prefrontal cortex of NK1R-/- mice in the light / dark exploration box

7.1. Introduction

The atypical DA response to *d*-AMP in cortico-striatal circuits of NK1R-/- mice, reported in Chapter 6, might offer an explanation for their behavioural abnormalities which echo those expressed by patients with ADHD. Nonetheless, ADHD is not a unitary disorder that is caused by a simple DA dysfunction, as drugs with primarily dopaminergic effects (e.g. the D1/D2 receptor agonist apomorphine) are not effective in relieving symptoms of this condition. Hence, it is possible that ADHD involves impaired functioning of other neurotransmitter systems in the brain in addition to, or as a result of, dysfunction of central DA systems.

The most recognized candidate is the LC-NA system, not least because drugs that are used to treat ADHD (e.g. psychostimulants, NAT blockers and α_2-adrenoceptor agonists) target central NA systems (see Section 1.4.2). Central NA transmission has critical modulatory influence on a variety of cognitive functions, such as arousal, vigilance and attention (Aston-Jones & Bloom, 1981; Devauges & Susan, 1990; Aston-Jones *et al*, 1991). It also appears to play a role in motor activation (see Berridge & Waterhouse, 2003), despite a long and controversial history of this concept. Early studies with monoamine depleting agents (e.g. reserpine) suggested a major role for the noradrenergic system in motor activity. Consistent with this view, later studies revealed that the locomotor stimulating effects of drugs of abuse (e.g. psychostimulants) rely on interactions between central NA and DA systems, possibly *via* α_1-adrenoceptors (Tessel & Barrett, 1986; Trovero *et al*, 1992; Darracq *et al*, 1998; also see Section 6.5.3). Based on the possible role of central NA systems in motor activity, altered noradrenergic transmission in NK1R-/- mice (Herpfer *et al*, 2005; Fisher *et al*, 2007; also see Chapter 4) might contribute to their hyperactivity, which is one of the key features of ADHD.

So far, it is already known that: a) NA transmission is abnormal in NK1R-/- mice; b) the PFC, whose functions are heavily modulated by NA, is implicated in ADHD (see Section 1.4.3); and c) the first-line ADHD medication, *d*-AMP, increased motor arousal of NK1R+/+ mice, but reduced the hyperactivity of NK1R-/- mice (see Chapter 5). Therefore, the **first aim** of this study was to find out whether the PFC NA response to *d*-AMP was abnormal in NK1R-/- mice, and whether this could explain their atypical motor response to this psychostimulant. To do so, the

effect of *d*-AMP on PFC NA efflux was compared, using *in vivo* microdialysis, in freely-moving NK1R+/+ and NK1R-/- mice in the LDEB (where a genotype difference in motor activity was observed).

In the **second part** of this study, PFC NA efflux of a new batch of NK1R+/+ and NK1R-/- mice was monitored for a *longer period* (see Section 7.3.2) while animals were confined in the light zone of the LDEB. This *modified LDEB protocol* was used because pilot studies revealed a genotype difference in NA efflux in the light zone which did not stabilize within the timeframe used earlier (*cf.* Fig 7.1 & 7.2). Moreover, whether or not this genotype difference was explained by disruption of NK1 receptors in NK1R-/- mice was tested, again, using the selective NK1R antagonist, RP 67580.

Also in this same randomized study (which used the *modified LDEB protocol*), cortical NA efflux was investigated in NK1R+/+ and NK1R-/- mice given the selective α_2-adrenoceptor antagonist, RX 821002. This is because somatodendritic α_2-adrenoceptors are desensitized in the mutants (Herpfer *et al*, 2005; Fisher *et al*, 2007). Activation of these autoreceptors in NK1R+/+, but not NK1R-/- mice, could confound any genotype difference in the modulatory effect of NK1 receptors on PFC NA efflux (as in Chapter 4). Finally, as before, whether or not any genotype difference(s) could be explained by a lack of functional NK1 receptors in the knockout was tested, using the NK1R antagonist, RP 67580.

7.2. Aim

***In vivo* microdialysis was used to:**

- **Investigate the effects of acute systemic administration of *d*-AMP on NA efflux in the PFC of NK1R+/+ and NK1R-/- mice in the LDEB.**
- **Characterize changes in NA efflux in the PFC of vehicle- and RX 821002 (α_2-adrenoceptor antagonist)-treated NK1R+/+ and NK1R-/- mice, which were confined in the light zone of the LDEB for a lengthened period. Whether any abnormalities of NK1R-/- mice could be due to a lack of their functional NK1 receptors was tested, using the selective NK1R antagonist, RP 67580.**

7.3. Protocols

7.3.1. The effects of *d*-AMP on NA efflux in the PFC

Surgery was performed under halothane anaesthesia, in which a microdialysis probe was inserted into the PFC (from bregma; in mm: AP +2.10, ML +1.0, DV -2.0). The next day, the microdialysis experiment was carried out. NK1R+/+ and NK1R-/- mice were randomly assigned to groups given an i.p. injection of either *d*-AMP (2.5 mg/kg) or saline (10 ml/kg) (see Fig 7.1).

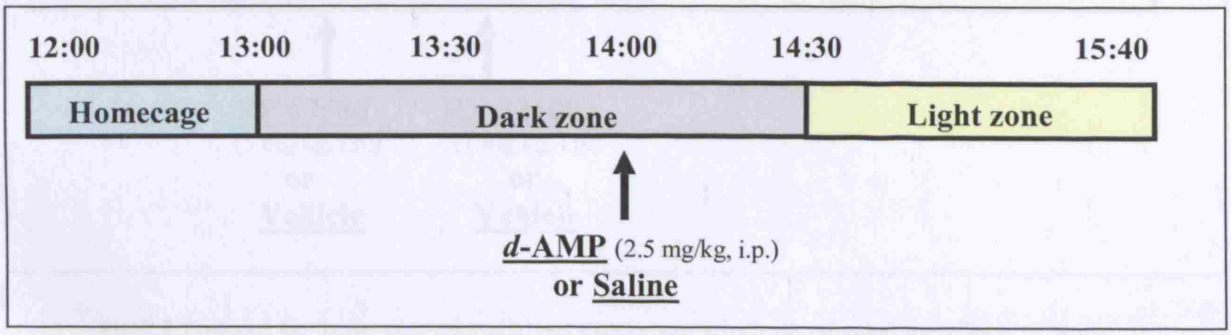


Fig 7.1 Protocol for the *in vivo* microdialysis experiment which investigated the effects of acute administration of *d*-AMP on NA efflux in the PFC of NK1R+/+ and NK1R-/- mice.

7.3.2. The effect of NK1R antagonism on PFC NA efflux in vehicle- & α_2-antagonist-treated mice

The protocol was similar to that described in Section 7.3.1. NK1R+/+ and NK1R-/- mice were randomly assigned to one of the 4 treatments:

Group	Drug 1	Drug 2
1).	Vehicle	Saline
2).	RP 67580 (5 mg/kg i.p.)	Saline
4)	Vehicle	RX 821002 (1 mg/kg i.p.)
5)	RP 67580 (5 mg/kg i.p.)	RX 821002 (1 mg/kg i.p.)

The selective α_2-adrenoceptor antagonist, RX 821002 ($Ki = 0.93 \pm 0.13$ nM in rats; Hudson *et al*, 1999), was administered at 1 mg/kg, because this dose has been found to increase NA efflux in the PFC of NK1R+/+ mice (Fisher *et al*, 2007).

Since the data revealed a gradual decline of NA efflux which did not reach a steady state within the timeframe mentioned in Section 7.3.1, microdialysis was extended until the decline stabilized (see Fig 7.2):

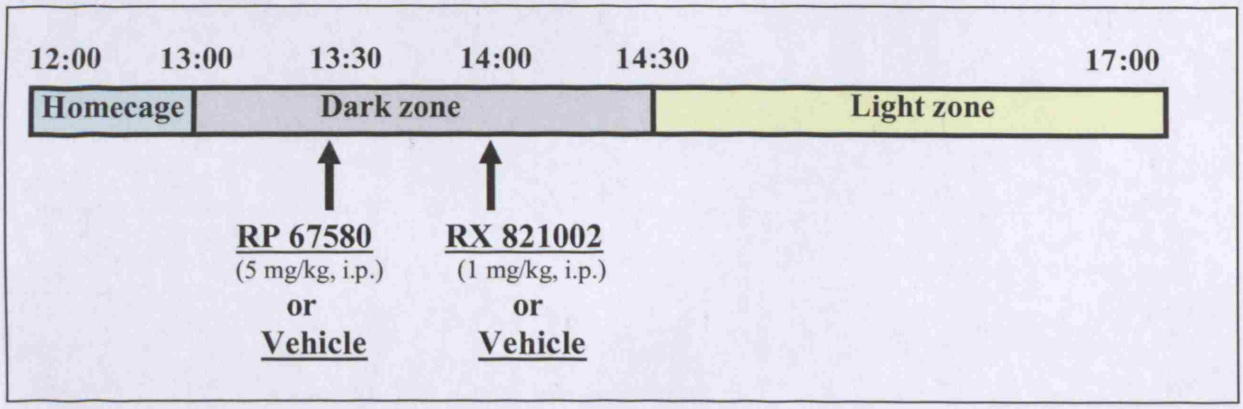


Fig 7.2 Protocol for the *in vivo* microdialysis experiment which investigated the effects of acute administration of the NK1R antagonist, RP 67580, and / or the α_2-adrenoceptor antagonist, RX 821002, on NA efflux in the PFC of NK1R+/+ and NK1R-/- mice.

7.4. Results

7.4.1. The effects of *d*-AMP on NA efflux in the PFC

Basal NA efflux in the PFC did not differ in freely-moving NK1R+/+ (9.4 ± 0.2 fmol / 20min) and NK1R-/- mice (8.9 ± 0.2 fmol / 20min) (see Fig 7.3 A).

Acute administration of *d*-AMP augmented cortical NA efflux in both genotypes (a main effect of 'Drug': $F_{1, 13} = 8.1$, $P < 0.05$). The extent to which this occurred did not differ in the two genotypes (see Fig 7.3):

In NK1R+/+ mice,

vs. basal:	Increased by +52%	$F_{1,3} = 13.2$	$P < 0.05$
vs. vehicle-treated NK1R+/+ controls:	Increased by +55%	$F_{1,6} = 5.6$	$P < 0.05$

In NK1R-/- mice,

vs. basal:	Increased by +51%	$F_{1,4} = 15.5$	$P < 0.05$
vs. vehicle-treated NK1R-/- controls:	Increased by +59%	$F_{1,7} = 7.5$	$P < 0.05$

Finally, as shown by the vehicle-treated NK1R+/+ and NK1R-/- mice, transferring animals to the novel light zone of the LDEB did not augment their PFC NA efflux.

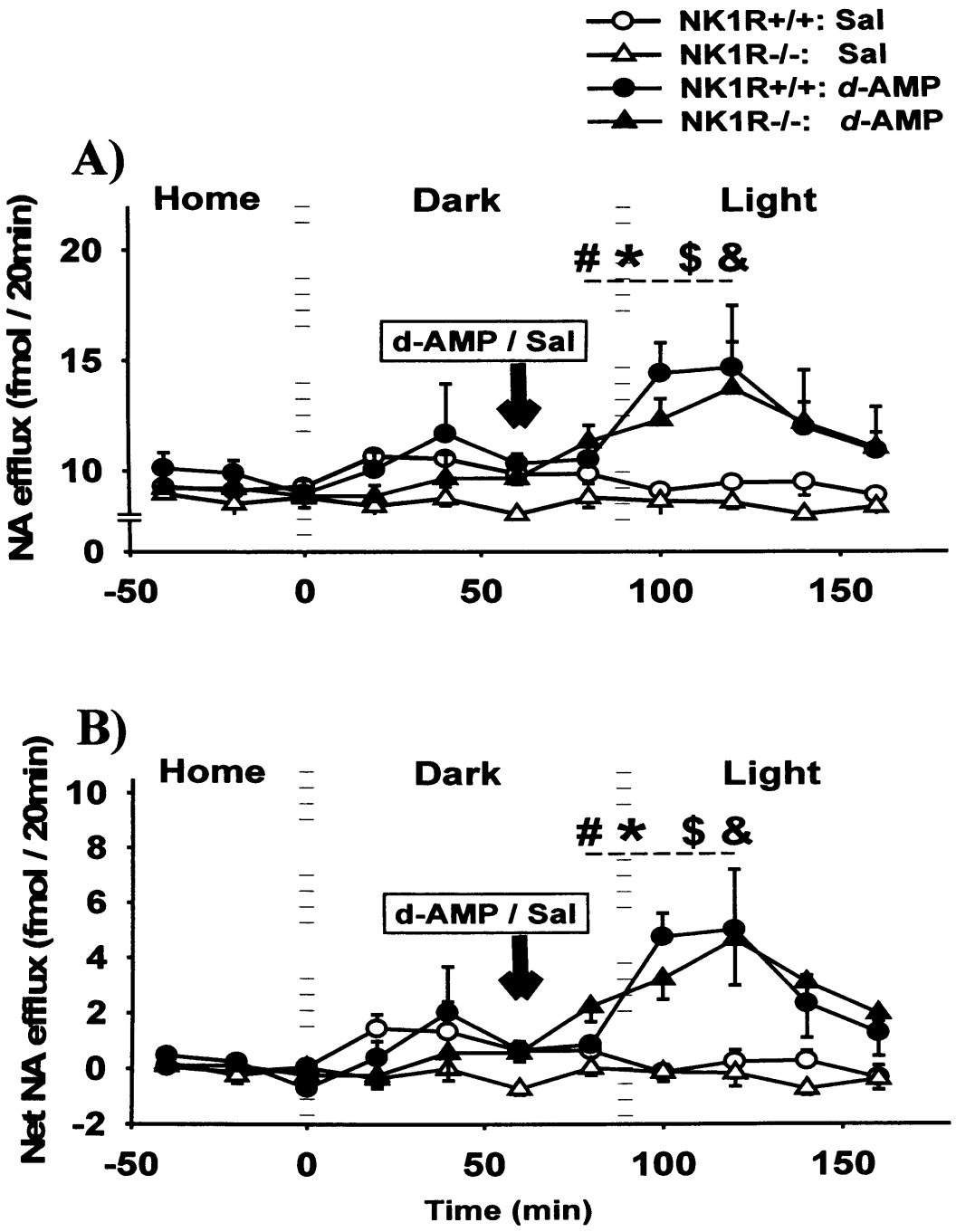


Fig 7.3 A) Raw data and B) net changes in the PFC NA response to *d*-AMP in NK1R+/+ and NK1R-/- mice in the LDEB (N = 4~5/group). Sal: Saline.

d-AMP-induced increase (T80~120) *vs.* Basal (T-40~0): NK1R+/+: # $P < 0.05$; NK1R-/-: * $P < 0.05$

At T80~120, *d*-AMP-treated mice *vs.* vehicle-treated controls: NK1R+/+: $ $P < 0.05$; NK1R-/-: & $P < 0.05$

7.4.2. The effect of NK1R antagonism on PFC NA efflux in the vehicle- & α_2-antagonist-treated mice

The changes in PFC NA efflux reported in this section were rather small (< 30%), but all of them reached the statistical criteria.

A 5-way ANOVA was performed. 'Genotype', 'RP 67580 (Drug 1)' and 'RX 821002 (Drug 2)' were regarded as between-subjects factors. 'Bin' and 'Time [T-40~0 (basals) *vs.* T200~240 (when efflux had stabilized)] were treated as within-subjects factors. A 'Genotype * RP 67580 * RX 821002' interaction was observed ($F_{1, 19} = 4.3$, $P < 0.05$). This indicated that the effects of the two drugs differed in the two genotypes.

For clarity, the data from the same fully randomized study are illustrated in 3 subsets (see Fig 7.4 ~ 7.6).

- ***The effects of NK1R antagonism on PFC NA efflux in the vehicle-treated mice***

When transferred to the light zone of the LDEB, cortical NA efflux in the vehicle-treated NK1R+/+ mice was sustained throughout the experiment, whereas that in the vehicle-treated NK1R-/- mice, or the wild-type pretreated with the NK1R antagonist RP 67580, gradually declined ('Genotype*RP 67580*Bin' interaction: $F_{1, 9} = 15.8$, $P < 0.01$). Paired comparisons of efflux during T200~T240 (when efflux had stabilized in the light zone) are listed below (RP: RP 67580; Sal: saline; Veh: vehicle):

NK1R-/- (Veh/Sal) *vs.* NK1R+/+ (Veh/Sal)	-13%	$F_{1,5} = 6.0$	$P < 0.05$
NK1R+/+ (RP/Sal) *vs.* NK1R+/+ (Veh/Sal):	-22%	$F_{1,5} = 17.7$	$P < 0.01$

Interestingly, in NK1R-/- mice pretreated with RP 67580, PFC NA efflux was rather labile immediately after the pretreatment. Although their NA efflux gradually decreased during T200~T240, the decline did not reach that seen in the vehicle-treated mutants within the experimental timeframe (see Fig 7.4).

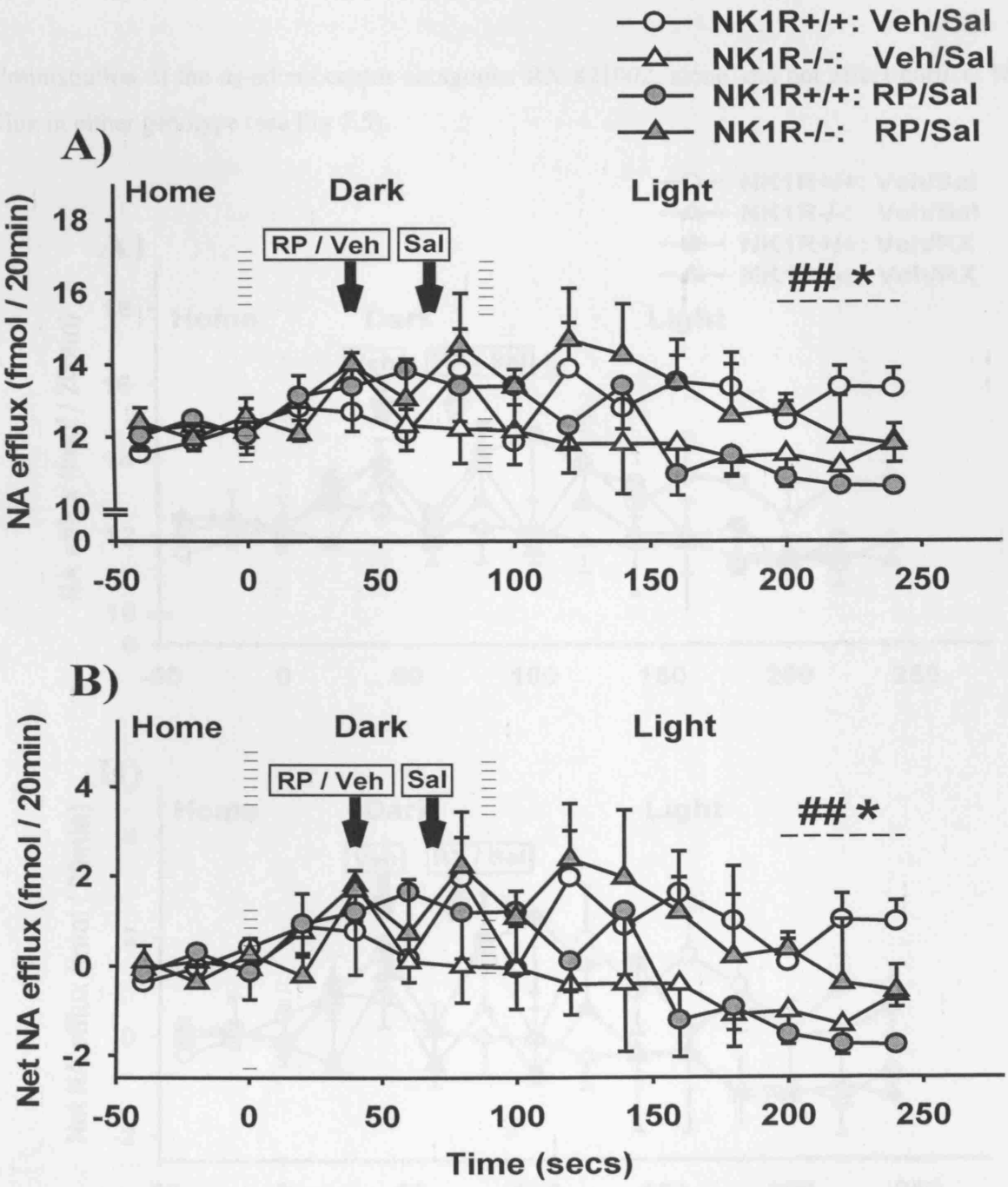


Fig 7.4 A) Raw data and B) net changes showing the effects of the NK1R antagonist, RP 67580, on PFC NA efflux in NK1R+/+ and NK1R-/- mice in the LDEB (N = 4~5/group). RP: RP 67580; Sal: saline; Veh: Vehicle.
NK1R+/+ (RP/Sal) *vs.* NK1R+/+ (Veh/Sal): T200~240: ## $P < 0.01$.
NK1R-/- (Veh/Sal) *vs.* NK1R+/+ (Veh/Sal): T200~240: * $P < 0.05$.

- ***The effects of NK1R antagonism on PFC NA efflux in the α_2-antagonist-treated mice***

Administration of the α_2-adrenoceptor antagonist RX 821002, alone, did not affect cortical NA efflux in either genotype (see Fig 7.5).

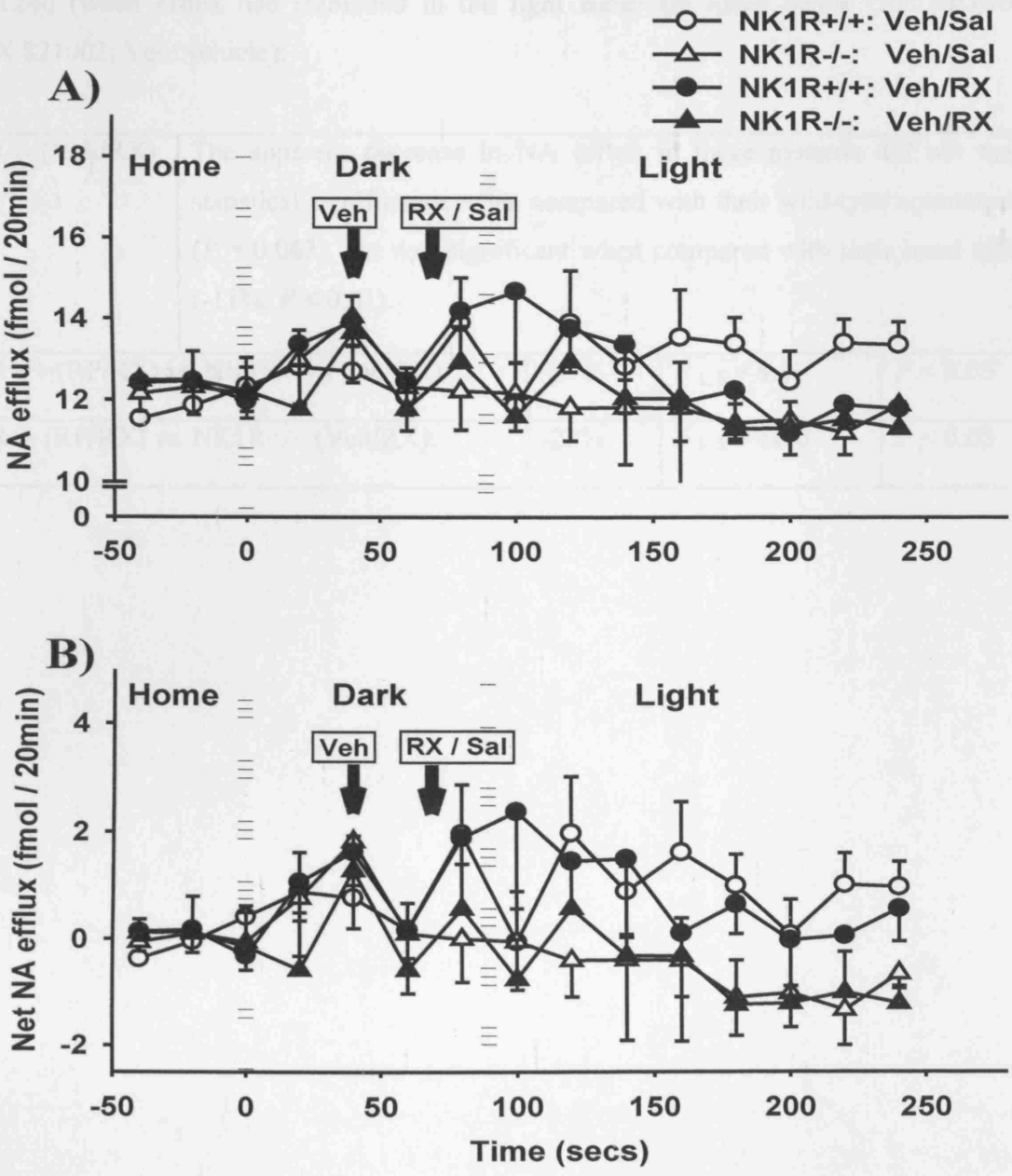


Fig 7.5 A) Raw data and B) net changes showing no significant changes in PFC NA efflux in NK1R+/+ and NK1R-/- mice in the LDEB, after administration of the α_2-adrenoceptor antagonist, RX 821002 (N = 4 ~ 5/group). RX: RX 821002; Sal: saline; Veh: vehicle.

Since RX 821002, alone, did not alter NA efflux in either genotype (see Fig 7.5), it is not surprising that the effect of the NK1R antagonist, RP 67580, was genotype-dependent in the RX 821002-treated mice ('Genotype*RP 67580*Bin' interaction: $F_{1, 10} = 6.2$, $P < 0.05$; see Fig 7.6), as in the vehicle-treated ones (see Fig 7.4). Paired comparisons of efflux during T200~T240 (when efflux had stabilized in the light zone) are listed below (RP: RP 67580; RX: RX 821002; Veh: vehicle):

NK1R-/- (Veh/RX)	The apparent decrease in NA efflux in these mutants did not reach statistical significance when compared with their wild-type counterparts ($P = 0.063$), but was significant when compared with their basal efflux (-11%; $P < 0.01$).		
NK1R+/+ (RP/RX) *vs.* NK1R+/+ (Veh/RX):	-15%	$F_{1,5} = 9.9$	$P < 0.05$
NK1R-/- (RP/RX) *vs.* NK1R+/+ (Veh/RX):	-23%	$F_{1,5} = 10.0$	$P < 0.05$

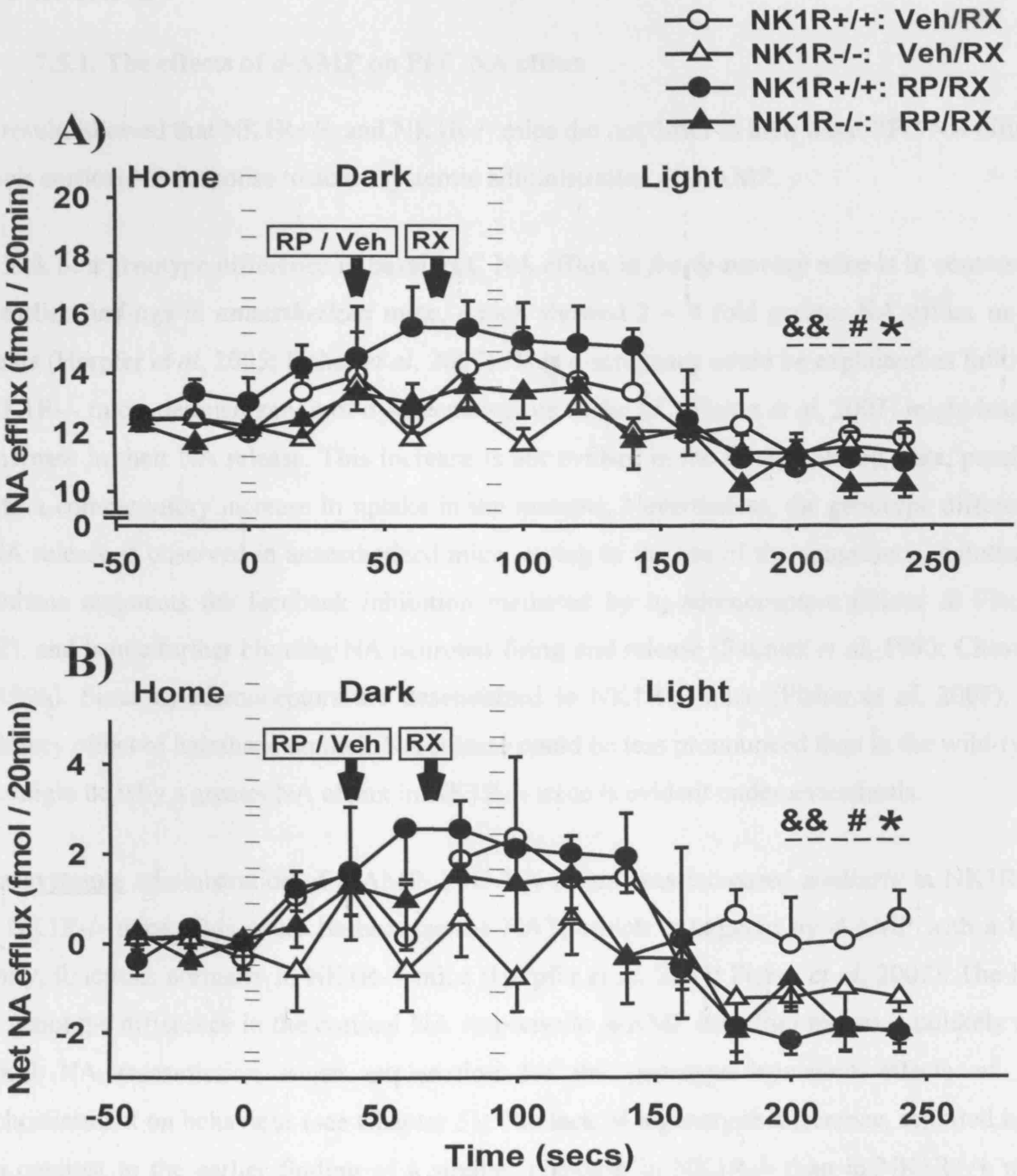


Fig 7.6 A) Raw data and B) net changes showing the effects of the NK1R antagonist, RP 67580, on PFC NA efflux in mice treated with the α_2-adrenoceptor antagonist, RX 821002 (N = 4~5/group). Veh: Vehicle; RP: RP 67580; RX: RX 821002.

NK1R-/- (Veh/RX): T200~240 *vs.* basal efflux: && $P < 0.01$

NK1R+/+ (RP/RX) *vs.* NK1R+/+ (Veh/RX): T200~240: # $P < 0.05$

NK1R-/- (RP/RX) *vs.* NK1R+/+ (Veh/RX): T200~240: * $P < 0.05$

7.5. Discussion

7.5.1. The effects of *d*-AMP on PFC NA efflux

The results showed that NK1R+/+ and NK1R-/- mice did not differ in their basal PFC NA efflux, or their cortical NA response to acute systemic administration of *d*-AMP.

The lack of a genotype difference in basal PFC NA efflux in ***freely-moving*** mice is in contrast to our earlier findings in ***anaesthetized*** mice, which showed 2 ~ 4 fold greater NA efflux in the mutants (Herpfer *et al*, 2005; Fisher *et al*, 2007). This discrepancy could be explained as follows. In NK1R-/- mice, desensitization of α_2-adrenoceptors in the LC (Fisher *et al*, 2007) might lead to an increase in their NA release. This increase is not evident in the freely-moving state, possibly due to a compensatory increase in uptake in the mutants. Nevertheless, the genotype difference in NA release is observed in anaesthetized mice, owing to the use of the anaesthetic, halothane. Halothane augments the feedback inhibition mediated by α_2-adrenoceptors (Bloor & Flacke, 1982), and hence further blunting NA neuronal firing and release (Saunier *et al*, 1993; Chave *et al*, 1996). Since α_2-adrenoceptors are desensitized in NK1R-/- mice (Fisher *et al*, 2007), the inhibitory effect of halothane on their NA release could be less pronounced than in the wild-type. This might be why a greater NA efflux in NK1R-/- mice is evident under anaesthesia.

After **systemic** administration of *d*-AMP, PFC NA efflux was increased *similarly* in NK1R+/+ and NK1R-/- mice. This could be because the NAT, which is targeted by *d*-AMP with a high affinity, functions normally in NK1R-/- mice (Herpfer *et al*, 2005; Fisher *et al*, 2007). The lack of a genotype difference in the cortical NA response to *d*-AMP therefore makes it unlikely that cortical NA transmission is an explanation for the genotype-dependent effects of this psychostimulant on behaviour (see Chapter 5). The lack of a genotype difference, reported here, is in contrast to the earlier finding of a smaller response in NK1R-/- than in NK1R+/+ mice following **local infusion** of *d*-AMP (see Section 4.4.1). This indicated that the effect of this drug at the cell body might mask that at the terminals.

Unlike the previous microdialysis study in rats from this lab (McQuade *et al*, 1999), the current results showed that exposure to the novel light zone of the LDEB did *not* augment PFC NA efflux in either NK1R+/+ or NK1R-/- mice. It is known that central NA transmission can be

increased by naturalistic stress, such as a novel environment (see Stanford, 1995), and the extent to which this is increased depends on the type of the stimuli. There is microdiaysis evidence from Dalley & Stanford (1995) that PFC NA efflux is increased in rats, as the light intensity of the novel arena is raised. Therefore, the present finding of a lack of a cortical NA response to novelty in mice could be due to the light intensity used (350 lux) being not aversive enough to elicit a NA response (*cf.* 2500 lux in the rat study: McQuade *et al*, 1999). It could also be attributed to the use of a different protocol in the current study.

7.5.2. The effect of NK1R antagonism on PFC NA efflux in the vehicle- and α_2-antagonist-treated mice

- ***The effects of NK1R antagonism on PFC NA efflux in the vehicle-treated mice***

PFC NA efflux in NK1R+/+ mice was stable throughout the experiment, but that in NK1R-/- mice and in NK1R+/+ mice pretreated with the NK1R antagonist, RP 67580, declined gradually after they were transferred to the light zone of the LDEB. This indicated that NK1 receptors are needed to maintain sustained NA transmission in the PFC.

Cortical NA is released from noradrenergic terminals projected from the LC. Using immunohistochemistry, high levels of immunoreactivity for NK1 receptors have been revealed on NA neurones in the LC in rats (Chen *et al*, 2000). Manipulation of these receptors, by substance P or NK1R antagonists, has been shown to influence the activity of LC-NA neurones. For instance, Guyenet and Aghajanian (1977) found that microiontophoretic application of substance P to the LC of anaesthetized rats excites most cells in this brain region, through locally expressed NK1 receptors. The increase in LC NA neuronal activity by substance P is also revealed *in vitro* from extracellular recordings from LC neurones contained in a pontine slice preparation (Cheeseman *et al*, 1983).

Nevertheless, findings of the effects of NK1R antagonists on the LC-NA system have been inconsistent. Millan *et al* (2001) found that the selective NK1R antagonist, GR 205171, ***potentiates*** NA cell firing in the LC of anaesthetized rats, and it also ***increases*** cortical NA efflux in freely-moving rats. This is supported by Maubach *et al* (2002), who found that another

selective NK1R antagonist L 760735, when given chronically, causes burst-firing of LC neurones in guinea-pigs *in vitro*. In contrast, Zocchi *et al* (2003) reported a ***lack of effect*** of GR 205171 on monoamine efflux in the frontal cortex of freely-moving CD1 mice. Nevertheless, the current data proposed a third theory: NK1R antagonism ***decreased*** PFC NA transmission.

The discrepancies between these studies could be due to differences in species and / or NK1R antagonists used, but more importantly it might be owing to differences in tonic activation of NK1 receptors in the animals under which their NA efflux is monitored. There is evidence in rats that, **during non-vigilant behaviours** (e.g. grooming), the LC neuronal activity is depressed by tonic inhibition impinging on the LC (Aston-Jones & Bloom, 1981). This inhibitory afferent is likely to be the GABAergic input from the nucleus prepositus hypoglossi (PrH) (Ennis & Aston-Jones, 1989). Immunohistochemical (Echevarri *et al*, 1997) and patch-clamping studies in rats (Bailey *et al*, 2004) showed that NK1 receptor are located on GABA neurones, and activation of these receptors increases GABA release. This population of NK1 receptors inhibitory to LC-NA neurones presumably displays greater tonic activity than the tonically 'silent' excitatory NK1 receptors on the NA cell bodies, as suggested by Millan *et al* (2001). Hence, when an NK1R antagonist is administrated in non-vigilant animals, the inhibition of the GABA-expressing NK1 receptors could be more pronounced than the inhibition of those on the LC-NA neurones. This would then remove the GABA inhibition of the LC, and lead to an increase in LC neuronal firing and NA release (see Fig 7.7 A).

The situation could be different when animals are **exposed to stress** (e.g. when confined in the novel light zone of the LDEB, as reported in Section 7.4.2). There is evidence that during stress, release of substance P is increased, which potentiates the LC neuronal activity and increases NA release from the PFC terminals (Ebner *et al*, 2004), *via* stimulation of NK1 receptors on the LC-NA neurones. Hence during stress, genetic ablation (in NK1R-/- mice) or pharmacological antagonism of NK1 receptors is expected to have a more pronounced effect in blocking these LC NK1 receptors (than those on the inhibitory GABA neurones), and consequently preventing the stress-induced increase in LC neuronal firing and NA release in the cortex (see Fig 7.7 B). This theory, which is supported by recent findings from Ebner & Singewald (2007), might explain the current finding of the lower PFC NA efflux in NK1R-/- and the wild-type given the NK1R antagonist, RP 67580, when they were confined in the novel light zone of the LDEB.

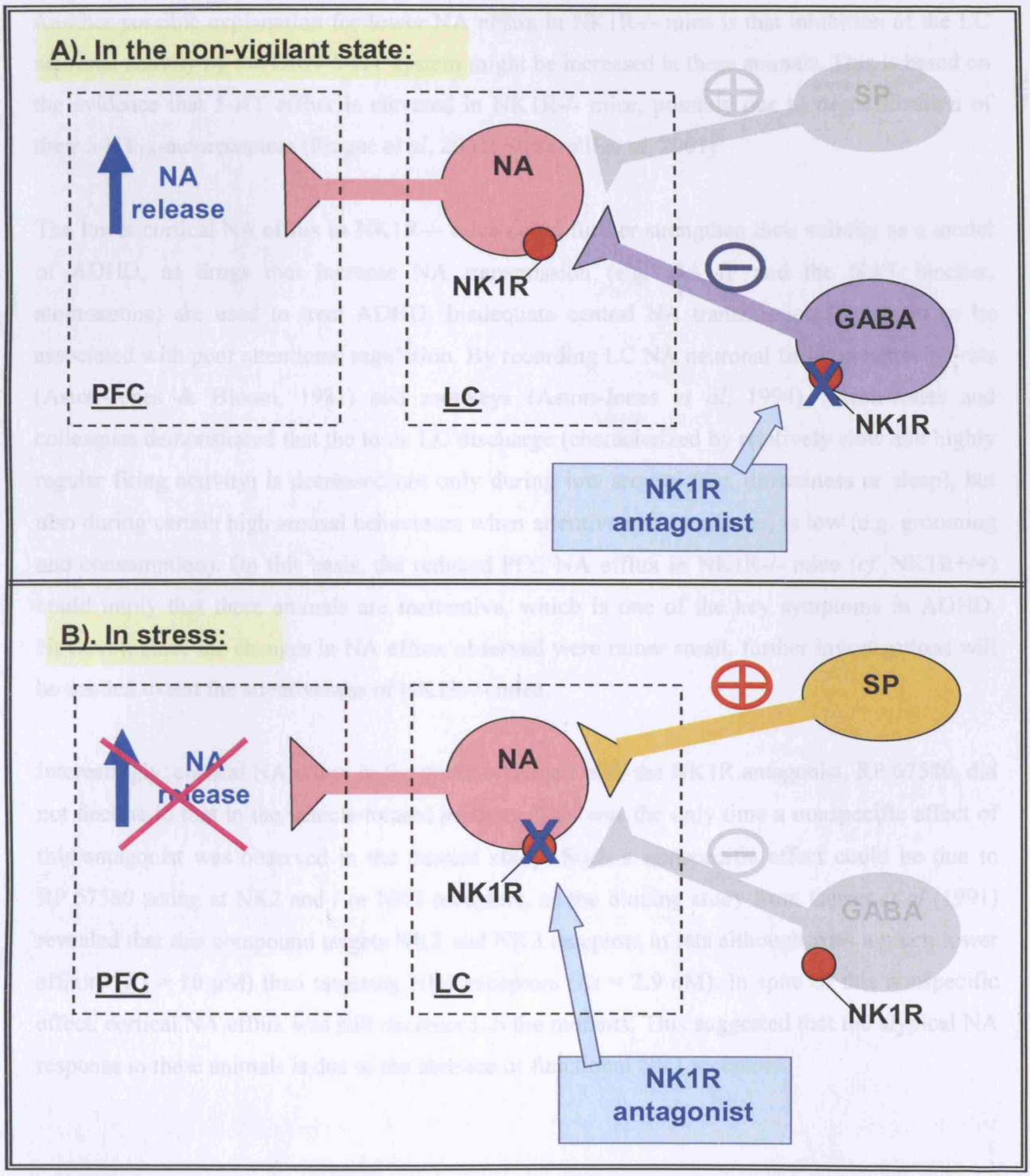


Fig 7.7 Diagram illustrating the possible mechanism of action of NK1R antagonists when administered in animals A) in the non-vigilant state; and B) when stressed.

Another possible explanation for lower NA efflux in NK1R-/- mice is that inhibition of the LC neuronal activity by the DRN-5-HT system might be increased in these animals. This is based on the evidence that 5-HT efflux is elevated in NK1R-/- mice, possibly due to desensitization of their 5-HT_{1A}-autoreceptors (Froger *et al*, 2001; Santerelli *et al*, 2001).

The lower cortical NA efflux in NK1R-/- mice could further strengthen their validity as a model of ADHD, as drugs that increase NA transmission (e.g. *d*-AMP and the NAT blocker, atomoxetine) are used to treat ADHD. Inadequate central NA transmission is thought to be associated with poor attentional regulation. By recording LC NA neuronal firing in behaving rats (Aston-Jones & Bloom, 1981) and monkeys (Aston-Jones *et al*, 1994), Aston-Jones and colleagues demonstrated that the tonic LC discharge (characterized by relatively slow and highly regular firing activity) is decreased not only during low arousal (e.g. drowsiness or sleep), but also during certain high arousal behaviours when attentiveness (vigilance) is low (e.g. grooming and consumption). On this basis, the reduced PFC NA efflux in NK1R-/- mice (*cf.* NK1R+/+) could imply that these animals are inattentive, which is one of the key symptoms in ADHD. However, since the changes in NA efflux observed were rather small, further investigations will be needed to test the attentiveness of NK1R-/- mice.

Interestingly, cortical NA efflux in the mutants treated with the NK1R antagonist, RP 67580, did not decline to that in the vehicle-treated mutants. This was the only time a nonspecific effect of this antagonist was observed in the current study. Such a nonspecific effect could be due to RP 67580 acting at NK2 and / or NK3 receptors, as the binding study from Garret *et al* (1991) revealed that this compound targets NK2 and NK3 receptors in rats although with a much lower affinity (Ki > 10 μM) than targeting NK1 receptors (Ki = 2.9 nM). In spite of this nonspecific effect, cortical NA efflux was still decreased in the mutants. This suggested that the atypical NA response in these animals is due to the absence of functional NK1 receptors.

- ***The effects NK1R antagonism on PFC NA efflux in the α_2-antagonist-treated mice***

α_2-Adrenoceptors on the NA cell bodies in the LC are desensitized in NK1R-/- mice (Fisher *et al*, 2007). Thus, it is important to find out whether or not this could contribute to the genotype difference in PFC NA efflux. The current results showed that there was no cortical NA response to the α_2-adrenoceptor antagonist, RX 821002, in either genotype. This therefore suggested that the lower PFC NA efflux in the mutants, observed when they were confined in the light zone of the LDEB, was not attributed to impaired functioning of their α_2-adrenoceptors. The fact that the NK1R antagonist, RP 67580, caused a similar reduction of NA efflux in vehicle- and RX 821002-treated NK1R+/+ mice, while having no effect in NK1R-/- mice, again proved that functional NK1 receptors are key to maintenance of NA transmission in the PFC.

Previously, the dialysis study by Fisher *et al* (2007) showed that RX 821002 (at 1mg/kg i.p.: same as that used here) increased cortical NA efflux in NK1R+/+, but not NK1R-/- mice. In contrast, the present study reported a lack of a PFC NA response to RX 821002 in both genotypes. In fact, it is not possible to compare the two studies, as they were performed under different environmental conditions. For instance, the current study was carried out in the LDEB, which could induce non-noxious environmental stress (McQuade *et al*, 1999), whereas the study by Fisher *et al* (2007) was performed in the relatively less stressful homecage. Moreover, mice used here received two injections (*cf.* only one injection in Fisher *et al* (2007)), which could have subjected the animals to greater stress. The more stressful protocol opted in the present study seemed to have resulted in rather *labile* NA efflux in all groups (including the control groups). This might have therefore masked any genotype-dependent effect of the α_2-adrenoceptor antagonist, RX 821002.

In conclusion, the current findings indicated that the decline in PFC NA efflux in NK1R-/- mice and the wild-type given the NK1R antagonist, RP 67580, was due to a lack of functional NK1 receptors in the mutants, rather than a genotype difference in function of α_2-adrenoceptors (but this does assume that *all* α_2-adrenoceptors have been blocked by the antagonist, atipamezole).

7.6. Summary

- **The effects of *d*-AMP on PFC NA efflux:**
  - **No difference in basal NA efflux in freely-moving NK1R+/+ and NK1R-/- mice.**
  - ***d*-AMP increased PFC NA efflux similarly in NK1R+/+ and NK1R-/- mice.**
    Hence, NA transmission in the PFC might not explain the genotype-dependent effects of *d*-AMP on the behaviour of NK1R+/+ and NK1R-/- mice in the LDEB.

- **The effects of NK1R antagonism on PFC NA efflux in the vehicle- and α_2-antagonist-treated NK1R+/+ and NK1R-/- mice:**
  - **When confined in the light zone of the LDEB, PFC NA efflux was sustained in NK1R+/+ mice, but declined progressively in NK1R-/- mice and in NK1R+/+ mice pretreated with the NK1R antagonist RP 67580 (although changes were rather small). Indicating that NK1 receptors are needed to maintain NA transmission in the PFC during arousal. This might be consistent with inattentiveness of ADHD.**
  - **The α_2-adrenoceptor antagonist, RX 821002, had no effect in either genotype. The NK1R antagonist, RP 67580, abolished the genotype difference in the RX 821002-treated mice as in the vehicle-treated ones.**
    Therefore, lower PFC NA transmission in NK1R-/- mice in the LDEB is likely to be due to disruption of their NK1 receptors, but not to desensitization of their α_2-adrenoceptors.

CHAPTER 8. General Discussion

Using *in vivo* microdialysis and the LDEB paradigm, this project revealed striking catecholaminergic and behavioural abnormalities in NK1R-/- mice.

Before characterizing the catecholaminergic response to drug treatments in NK1R+/+ and NK1R-/- mice, it was important to establish whether or not their transmitter release was impulse-dependent. This was confirmed to be the case by the microdialysis studies in **Chapter 3**, as infusion of the Ringer's solution that contained 80 mM $[K^+]_o$ into the PFC enhanced cortical NA efflux in both NK1R+/+ and NK1R-/- mice, and their NA response to K^+ was abolished by removal of Ca^{2+} from the perfusion medium.

Also in **Chapter 3**, genotype differences in NA efflux were observed in response to infusion of either two successive high-K^+ pulses or one high-K^+ followed by one Ca^{2+}-removal pulse. NK1R-/-, but not NK1R+/+, mice had sustained NA response to the *second* K^+ stimulation. This suggested that NK1R-/- mice might have: (i) a larger releasable pool of NA (e.g. increased expression of VMAT2 which enhances the vesicular monoamine storage capacity); and / or (ii) more efficient excitation-release coupling (e.g. increased expression of the nerve terminal protein, SNAP-25, which enables a more rapid release process).

Moreover, removing Ca^{2+} from the Ringer's solution at the *second* pulse reduced the NA response to 80 mM $[K^+]_o$ stimulation in NK1R-/- mice. In contrast, in NK1R+/+ mice, the effect of Ca^{2+} depletion was observed only when applying a single-pulse. This suggested that, in the wild-type, the response to Ca^{2+} depletion at the *second* pulse was masked by a lack of the NA response to the *second* K^+ pulse. Therefore, it seemed that the response to Ca^{2+}-depletion is more evident in NK1R-/- than in NK1R+/+ mice. A possible explanation for this is that, in NK1R-/- mice, α_2-adrenoreceptors in the LC are desensitized (Fisher *et al*, 2007), which reduces the feedback inhibition of their impulse-evoked NA release. Consequently, the greater NA release in the mutants might engender a greater demand for Ca^{2+} to maintain the release process.

After establishing the impulse-dependency of transmitter release, NK1R+/+ and NK1R-/- mice were compared for their NA response to local infusion of the monoamine-releasing agent, *d*-AMP, in the PFC (**Chapter 4**). This was investigated because Murtra *et al* (2000a) reported that NK1R-/-

mice, unlike their wild-types, do not respond to the rewarding effect of *d*-AMP in the conditioned place preference (CPP) test. Using *in vivo* microdialysis, NA efflux in the PFC was found to be increased dose-dependently by *d*-AMP in NK1R+/+ mice, whereas it was increased only at the lower drug concentration in NK1R-/- mice. Since autoinhibition of NA release by α_2-adrenoceptors is impaired in the mutants (Herpfer *et al*, 2005; Fisher *et al*, 2007), the increased NA release in these animals might lead to greater competition between extracellular NA and the psychostimulant. This could thus result in the drug not being able to be taken up into nerve terminals to exert its releasing effect in NK1R-/- mice, hence their smaller NA response to *d*-AMP.

Moreover, previous microdialysis studies in rats revealed that at low, but not high, concentrations of *d*-AMP, the drug-induced impulse-independent release of NA can be masked by activation of α_2-adrenoceptors (Géranton *et al*, 2003). These receptors are desensitized in NK1R-/- mice (Fisher *et al*, 2007). Therefore, the next experiment compared the two genotypes for their NA response to local infusion of *d*-AMP in the PFC ***after taking out the confounding factor, α_2-adrenoceptors.*** This was implemented by pretreating NK1R+/+ and NK1R-/- mice with a selective α_2-adrenoceptor antagonist, atipamezole, systemically. The results showed that, at the lower dose of *d*-AMP, the genotype difference was *still* evident in the atipamezole-pretreated animals. This indicated that the difference in the PFC NA response to *d*-AMP in NK1R+/+ and NK1R-/- mice, at low drug concentrations, was not due to a genotype difference in α_2-adrenoceptor function. In contrast, at the higher dose of *d*-AMP, the genotype difference was *abolished* in the pretreated animals. This might be attributed to the overwhelming impulse-independent release of NA by *d*-AMP at high drug concentrations, which masked any compensatory effects of α_2-adrenoceptors, as suggested by the findings in rats (Géranton *et al*, 2003). In fact, it is the low drug doses that are of clinical relevance in treating disorders like ADHD, as higher doses usually lead to stereotypy in rodents and increased arousal in humans.

In **Chapter 4**, the effects of local infusion of another psychostimulant, MPH (which has a greater affinity for DAT than NAT), on PFC DA and NA efflux was also tested in NK1R+/+ and NK1R-/- mice. This is because MPH, as with *d*-AMP, produced different effects on locomotor activity in the two genotypes (see Chapter 5). We therefore investigated whether this could be associated with any genotype-dependent catecholaminergic responses to this drug. The results showed that the

cortical NA response to MPH did not differ in NK1R+/+ and NK1R-/- mice. However, there were marked genotype differences in DA transmission: (i) NK1R-/- mice displayed lower basal DA efflux in the PFC (which was also observed in the experiments described in Chapter 6). This might echo the hypofrontality in ADHD patients (Zametkin *et al*, 1990; Ernst *et al*, 1998; Rubia *et al*, 1999); (ii) MPH caused a moderate increase in cortical DA efflux in the mutants at the low dose (10 μM). This is consistent with the report that psychostimulants relieve hypofrontality (Daniel *et al*, 1991). In fact, activity of the frontal cortex is also increased by guanfacine, another ADHD medication. This is based on a recent imaging study that this drug increases the blood oxygenation level dependent (BOLD) response in the cortical region in rats (Easton *et al*, 2006). Finally, the study described in Chapter 4 also showed that MPH increased PFC DA response in NK1R+/+ mice at the high dose (100 μM). However, the response was rather transient, possibly due to some inhibitory factor(s).

Having found that the NA response to local infusion of *d*-AMP in the PFC is atypical in NK1R-/- mice (see Chapter 4), whether their locomotor response to systemic administration of this drug was also abnormal was tested in **Chapter 5**. This behavioural study was performed in the LDEB, in which hyperactivity of NK1R-/- mice has been consistently reported by our lab (Herpfer *et al*, 2005; Fisher *et al*, 2007). A striking genotype difference was observed in the motor response to *d*-AMP: the drug increased locomotion of NK1R+/+ mice, but reduced the hyperactivity of NK1R-/- mice. This echoed the situation in ADHD, where psychostimulants have a calming effect in individuals with this disorder. Similar changes were also observed with MPH (another first-line ADHD medication), which caused NK1R+/+ mice to be hyperactive, while made the locomotor activity of NK1R-/- mice resemble that of the vehicle-treated NK1R+/+ controls. Collectively, these behavioural data suggested that NK1R-/- mice could be a novel rodent model of ADHD.

Interestingly, in this project, the genotype differences in specific behaviours (e.g. *locomotor activity* and *risk assessment behaviours*) were more robust in the light zone of the LDEB than the dark zone where animals were habituated to before the test. Since hyperactivity of NK1R-/- mice was observed during the 1st 30 min habituation to the dark zone, it is thought that the lack of a genotype difference in this compartment during the test is because this compartment is no longer novel or stressful to the animals. Further, habituation to the dark zone was slower in NK1R-/- than in NK1R+/+ mice. This might consolidate the validity of NK1R-/- mice as a model of ADHD,

since impaired habituation to novelty has been reported in several ADHD models, such as the DAT knock-out or knock-down mouse (Gainetdinov *et al*, 1999; Zhuang *et al*, 2001) and the thyroid receptor β PV knock-in mouse (Siesser *et al*, 2005). However, further considerations should be taken, as it is not yet clear whether this is also the case in humans with ADHD.

Before further testing the validity of this model, it was crucial to verify whether these genotype differences in behaviour were due to disruption of NK1 receptors in NK1R-/- mice, or to developmental / compensatory change(s). To do so, the behavioural response to *d*-AMP was investigated in another batch of NK1R+/+ and NK1R-/- mice, which were pretreated with a selective NK1R antagonist, either RP 67580 or L 733060 (see **Chapter 5**). It was found that both NK1R antagonists made the behaviour of NK1R+/+ mice resemble that of NK1R-/- mice, but had no nonspecific effects in the mutants. This confirmed that the behavioural abnormalities of NK1R-/- mice were explained by their lack of functional of NK1 receptors.

The validity of NK1R-/- mice as a model of ADHD was then tested neurochemically, using *in vivo* microdialysis in the LDEB. Most literature on ADHD points to abnormal DA transmission in cortico-striatal circuits as the major causal factor for this disorder (see Madras *et al*, 2005; Pliszka, 2005; van der Kooij & Glennon, 2007). Therefore, basal DA transmission and the DA response to systemic administration of *d*-AMP were investigated in the ***PFC*** and the ***dorsal striatum*** of NK1R+/+ and NK1R-/- mice, while they were in the LDEB (see **Chapter 6**):

Basal DA efflux in the PFC was again lower in NK1R-/- than in NK1R+/+ mice (as with that reported in Chapter 4). This might explain why basal DA efflux in the dorsal striatum was *not* lower in the mutants (given that DA released in the PFC reduces DA transmission in the subcortical areas, *via* inhibition of the excitatory cortical glutamatergic projections to the latter brain regions: Ventura *et al*, 2004; also see Section 1.2.3.2).

Systemic administration of *d*-AMP augmented DA efflux in the ***dorsal striatum*** of NK1R+/+ mice, but not that of NK1R-/- mice. Since striatal DA transmission modulates the motor response to psychostimulants (see Section 1.2.3.1), these findings might explain why *d*-AMP caused motor arousal in NK1R+/+, but not NK1R-/- mice. It is also known that the *d*-AMP-induced increase in

striatal DA transmission could account for the stereotypic effect of the drug (Fibiger *et al*, 1973; Kelly *et al*, 1975; Arnt 1985), but this occurs only at *high* drug concentrations (see Rebec, 2006). The results reported in Chapter 5 showed that the dose used in this project (2.5 mg/kg i.p.) increased locomotor activity of NK1R+/+ mice without causing stereotypy. On the other hand, in the ***PFC***, *d*-AMP did not affect DA efflux in either genotype. This could be due to efficient clearance processes in this brain region and / or existence of factor(s) holding down the response.

Pretreatment with RP 67580 abolished all genotype differences in regulation of DA transmission in the PFC and the dorsal striatum, by turning NK1R+/+ into NK1R-/- mice. This suggested that these dopaminergic abnormalities in the mutants were attributed to disruption of their functional NK1 receptors.

The current findings of abnormal DA transmission in cortico-striatal circuits in NK1R-/- mice greatly support the validity of this ADHD model, given that central DA systems play a crucial role in neuromodulation of frontal-executive function (e.g. spatial working memory; see Robbins 2000) and motor activity (see Rebec, 2006) in both animals and humans. Interestingly, while *d*-AMP *reduced* the hyperactivity of NK1R-/- mice in the LDEB (see Chapter 5), it did not reduce DA transmission in the dorsal striatum of NK1R-/- mice (see Chapter 6). Therefore, factors other than striatal DA transmission could also be involved in the calming effect of *d*-AMP. NA transmission in the PFC could be a candidate in this case, not least because it is targeted by many ADHD medications, such as psychostimulants (*d*-AMP and MPH) and α_2-adrenoceptor agonists (clonidine and guanfacine) (see Pliszka, 2005; Arnsten, 2006). Moreover, NA is of fundamental importance in modulating arousal (Aston-Jones *et al*, 1991; see Berridge & Waterhouse, 2003), which superimposed on the increased locomotion by psychostimulants. Using microdialysis, Berridge & Stalnaker (2002) found that increased PFC NA efflux contributes to the low-dose behavioural effects of amphetamine-like stimulants in rats, including the arousing-enhancing actions. Therefore, in **Chapter 7**, the cortical NA response to systemic injection of *d*-AMP was compared in NK1R+/+ and NK1R-/- mice. The findings revealed that *d*-AMP elicited a similar NA response in both genotypes. This therefore *rules out* the possibility that PFC NA transmission might contribute to the different effects of *d*-AMP on the behaviour of NK1R+/+ and NK1R-/- mice.

Also in **Chapter 7**, NA efflux in the PFC progressively declined in NK1R-/-, but not in NK1R+/+ mice, when they were confined in the light zone of the LDEB. The genotype difference was abolished by the NK1R antagonist, RP 67850, which reduced NA efflux in NK1R+/+ mice, only. This indicated that functional NK1 receptors are required to maintain cortical NA transmission during stress / exposure to novelty. Lower PFC NA efflux in NK1R-/- mice could be consistent with inattentiveness in ADHD, as Aston-Jones and colleagues found that insufficient NA transmission is evident not only during low arousal (e.g. drowsiness and sleep), but also during certain high arousal behaviours when attentiveness is low (e.g. grooming and consumption) (see Aston-Jones *et al*, 1991; 1994; 1999; Aston-Jones & Cohen, 2005). Nevertheless, further investigations are needed to confirm the relevance of this NA deficit in NK1R-/- mice to inattentiveness of ADHD, because the changes were very small (within 30%), and also because inattentiveness could be associated with not just a deficient, but also excessive NA transmission (see Berridge, 2001).

In addition, **Chapter 7** revealed that the difference in PFC NA efflux was still evident in NK1R+/+ and NK1R-/- mice pretreated with a selective α_2-adrenoceptor antagonist RX 821002, but was abolished by the NK1R antagonist RP 67580 (which made NK1R+/+ resemble NK1R-/- mice). Collectively, these results suggested that the genotype difference in cortical NA efflux during stress was unlikely to be due to a difference in α_2-adrenoceptor function, but seemed to be caused by impairment of functions of NK1 receptors in the mutants.

Genetic studies have revealed several polymorphic genes in ADHD. Most of these polymorphisms suggest the contribution of abnormal monoamine functioning to pathophysiology of ADHD. The current project has revealed altered DA and NA transmission in NK1R-/- mice. NK1 receptors are located upstream of these catecholamine systems. This topographical arrangement makes it possible that NK1 receptors modulate central monoamine systems that are implicated in ADHD. In NK1R-/- mice, functional ablation of NK1 receptors might thus lead to imbalanced monoamine transmission, hence resulting in development of ADHD symptoms.

In conclusion, all the behavioural and neurochemical abnormalities in NK1R-/- mice (see Table 9.1) strongly support their use as a novel rodent model of ADHD. It follows that drugs that increase activation of NK1 receptors could offer a novel class of treatment for this disorder.

A).

	NK1R-/- mice		ADHD
Behaviour	**Hyperactivity** (also Herpfer et al, 2005; Fisher et al, 2007)	√	**Hyperactivity**
	↓ Hyperactivity by *d*-AMP & MPH	√	**Calm after psychostimulants**
	Impulsivity (pilot studies)	√	**Impulsivity**
Neurochemistry	**↓ NA efflux in the PFC**	√	**Inattentiveness**
	↓ DA efflux in the PFC	√	**Hypofrontality** (Rubia et al,1998)
	No DA response to *d*-AMP in the striatum	√	**Blunted arousal response to *d*-AMP**

B).

	NK1R-/- mice		ADHD
Behaviour	**Impaired motor co-ordination & learning in the rotarod test (De Felipe et al (unpublished))**	√	**Accident-prone / 'clumsy'**
	Do not develop CPP to *d*-AMP (Murtra et al, 2000a)	√	**↓ dependence on *d*-AMP**
Neurochemistry	**α_2-Adrenoceptors on NA cell bodies in the LC are desensitized (Fisher et al, 2007)**	√	**α_2-adrenoceptor agonists are used to treat ADHD (e.g. clonidine & guanfacine)**

Table 8.1 Comparisons between abnormalities in NK1R-/- mice and symptoms of ADHD. A). The behavioural and neurochemical abnormalities of NK1R-/- mice reported in this project. B). Abnormalities of NK1R-/- mice reported by other studies.

Appendix I

Genotype verification

Step 1. Sample preparation:

- Mouse tail snip (5 mm) is removed with a clean scalpel blade and placed in a sterile labeled 1.5 ml eppendorf tube.
- 750 µl tail lysis buffer and 22.5 µl proteinase K (Sigma) are added to each sample and vortexed briefly.

> Tail lysis buffer contains 50 mM Tris pH 8 (Sigma), 100 mM EDTA pH 8 (Sigma), 10 mM NaCl (BDH) and 1% SDS (Sigma).

- Samples are then incubated in water bath at 55°C overnight. If necessary, samples are stored at -20°C the next day.
- The frozen samples are defrosted, then vortexed briefly before centrifugation for 3 min at 13,000 rpm. The supernatant is then transferred to a fresh labeled 1.5 ml eppendorf tube, into which 200 µl protein precipitation solution (Puregene, Minneapolis, USA) is added. This is followed by vortexing for 20 secs and centrifugation for 3 min at 13,000 rpm.
- Supernatant is then poured into a new eppendorf tube, containing 600 µl isopropanol (Sigma), and mixed by gentle inversion. Centrifuge as before.
- Supernatant is removed. The pellet is washed in 300 µl 70% ethanol and mixed by flicking tube, followed by centrifuging as before.
- Supernatant is removed and the tubes are left open to air dry for a couple of hours (to allow the ethanol to evaporate).
- 250 µl TE (10 mM Tris pH 8 (Sigma), 1 mM EDTA pH 8 (Sigma)) is added to each sample and mixed and spinned.
- The precipitate in each sample is dissolved overnight at 4°C.

Step 2. Polymerase Chain Reaction (PCR)

- Making up master mix (volumes shown are for 30 samples):

• Ultra pure water (1056 µl) • *Taq* DNA polymerase 10 x reaction buffer (165 µl; Promega, Southampton, U.K.) • $MgCl_2$ (99 µl; Promega) • dNTP (33 µl; Promega) • 3 primers: NeoF[1], NK1-F[2], NK1-R[3] (33 µl each; Sigma) • *Taq* DNA polymerase (12 µl; Promega)

- 5 µl DNA sample is added into each PCR tube. Controls are also prepared (two PCR Reg tubes for +/+ and -/-, and one tube for ultra pure water). These samples are then mixed with 35 µl master mix.
- Tubes are placed in a PCR temperature cycler (PTC-100 Programmable Thermal Controller, MJ Research, Boston, U.S.A.). The following programme is set to run:

• 95°C for 5 min	
• 60°C for 30 sec	
• 72°C for 30 sec	
• 94°C for 30 sec	x 34
• 60°C for 30 sec	
• 72°C for 5 min	

- After PCR, 10 µl loading buffer is added to each sample and mixed

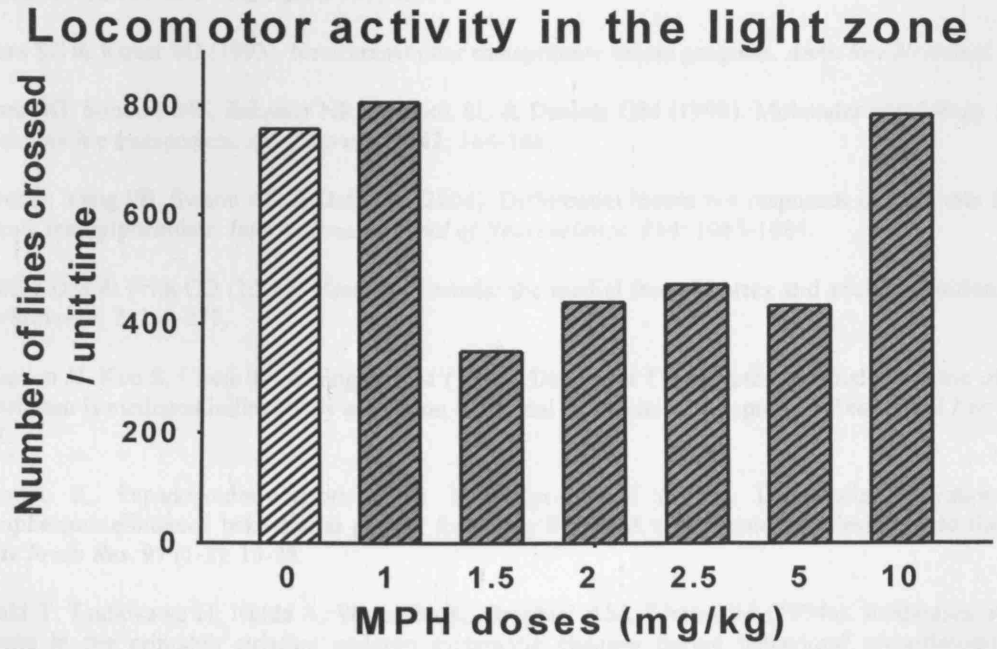
Loading buffer contains 0.25% bromophenol blue (Sigma), 0.25% xyelene cyanol FF (Sigma) and 30% glycerol (BDH).

- 11 µl of each sample is run on a 2% agarose gel in the TBE buffer (National Diagnostics, Hull, U.K.) containing 10 µl ethidium bromide (Sigma) at 120 mV for approximately 1 h.
- DNA bands can then be visualized and photographed under ultraviolet transillumination.

Appendix II

The locomotor response of NK1R-/- mice to a range of MPH doses in the LDEB

To investigate whether the hyperactivity of NK1R-/- mice in the light zone could be significantly abolished by MPH, just as that by *d*-AMP, a supplementary pilot study was performed. Using the same protocol (see Section 5.3.1), a new batch of NK1R-/- mice were challenged with a range of MPH doses (0, 1, 1.5, 2, 2.5, 5 and 10 mg/kg i.p.). The results showed that with exception of 1 and 10 mg/kg (the lowest and the highest doses, respectively), all the doses caused a reduction of the hyperactivity of NK1R-/- mice (N = 1; see Fig below).



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